The application of macroscopic enzyme histochemistry for the gross identification of early human myocardial infacts.

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THE APPLICATION OF MACROSCOPIC ENZYME HISTOCHEMISTRY FOR THE GROSS IDENTIFICATION OF EARLY HUMAN MYOCARDIAL INFARCTS

THESIS

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A B S T R A C T

The accurate diagnosis of acute myocardial infarction at necropsy frequently poses a problem to the pathologist, particularly when sufficient time has not elapsed for the development of detectable morphological changes in the myocardium. For this purpose human hearts obtained at necropsy were subjected to enzyme and non-enzyme histochemical reactions for the early macroscopic or microscopic identification of the lesion.

Various enzyme systems in the heart were examined in the gross. These included dehydrogenases, diaphorases, cytochrome oxidase, monoamine oxidase, myoglobin peroxidase, phosphorylase, glutamic-oxaloacetic transaminase, creatine phosphokinase, leucine aminopeptidase, non-specific esterase, and acid phosphatase.

On the basis of this study, dehydrogenases and diaphorases based on nitroblue tetrazolium as a redox indicator were found to be the most reliable. The macroreaction depends upon enzymatic reduction of nitroblue tetrazolium by normal heart muscle and the focal absence of such staining in necrosis and infarction. The conditions were determined that provide the maximum nitroblue tetrazolium macroreaction, and were applied in this study. It was observed that the non-specific dehydrogenase incubating medium using the heart's own endogenous substrate, with added NAD (nicotinamide adenine dinucleotide; coenzyme I) and the respiratory chain inhibitor, cyanide, is the most sensitive in depicting areas of recent myocardial damage. The use of NAD and cyanide for the gross detection of the lesion is a new modification introduced in the present study.

Normal heart muscle contains substrates, coenzymes and enzymes. It is suggested that the coenzyme NAD and the enzyme NAD tetrazolium reductase are the first to be depleted from the injured myocardium. This could be of clinical significance in the diagnosis and prognosis of acute myocardial infarction.

The persistence of the tetrazolium dehydrogenase reaction when NAD is added to the incubating medium leads one to consider that the reaction, in fact, depends upon NADH (reduced nicotinamide adenine dinucleotide) tetrazolium reductase within the tissue. The addition of phenazine methosulphate (PMS) to by-pass the tissue reductase or diaphorase in the dehydrogenase macroreaction resulted in reduction of nitroblue tetrazolium in the incubating medium and non-selective staining of the heart. It seems that PMS accelerated the transfer of electrons to the tetrazolium salt in solution rather than in situ.

Phloxine tartrazine (Lendrum, 1947) was used here as an aid in the microscopic identification of early human myocardial infarction in microscopic tissue sections. The method revealed areas of recent myocardial damage long before myocardial fibres showed any histologic evidence of necrosis. It proved to be more valuable in microscopical application than conventional non-enzyme histochemical methods.

Waviness of myocardial fibres has been reported to be an early morphologic index of acute myocardial infarction. However, it was found here to be non-specific, and equally distributed amongst infarcted and control hearts. It was frequently also seen in the autolysed heart.

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CHAPTER I

INTRODUCTION

The accurate recognition of early myocardial infarcts in the gross at necropsy frequently poses a problem. If the symptoms of acute myocardial infarction were of short duration, often the infarct cannot be detected with certainty, because unequivocal gross morphological changes characteristic of the infarct are usually considered to take a relatively long time to become apparent. In the absence of gross changes, the involved area of myocardium can be missed when random blocks are taken for histologic examination. In addition, it is well known that the classical histologic alterations of myocardial infarction, using routine histologic techniques, require some time to become readily apparent.

The morphological alterations of acute myocardial infarction

The pathological alterations of myocardial infarction, its early gross and microscopic changes, have been extensively studied previously both in human autopsy material and experimentally. Weigert (1880) showed that infarction of the heart was analagous to infarction in other organs. He described both the macroscopic and microscopic appearances of acute myocardial infarction and its healing process. He reported that the heart muscle became dry and yellow following coronary artery occlusion. Histologically there was loss of muscle nuclei and the healing process consisted in the removal of necrotic muscle and its replacement by granulation, and eventually by scar tissue.

Levine and Brown (1929) in their monograph on the clinical features of coronary thrombosis, were the first to publish a detailed attempted correlation between the age of the infarct and the gross histologic findings. The time of onset of myocardial infarction was determined from the history and the character of the pain in twenty-four out of forty-six cases in their series where an autopsy was performed. The earliest interval between the onset and death was twenty-four hours. Grossly, they observed that, during the first day, infarcted muscle appeared deeply red due to extravasation of blood into the muscle. This feature was particularly marked during the first three to four days. Associated with extravasation of blood, there was variable degree of yellowish mottling of the muscle which varied with the degree of necrosis and was softer in consistency than the uninvolved muscle. They also found that necrosis of the heart muscle, which was prominent from the beginning, predominated from the fourth day to the end of the third week. Repair by connective tissue proliferation started on the sixth day and by the fifth week sufficient scarring had occurred to prevent rupture of the heart, and by the eighth week, full scarring was present. These observations have been the foundation of the traditional six weeks rest insisted upon by the physician in patients with acute myocardial infarction. Microscopically, they observed that oedema and haemorrhage were the earliest manifestation of the acute attack and appeared during the first few hours to be shortly followed by necrosis of the muscle fibres and by leucocytic infiltration. Collagen repair then followed, and eventually was transformed into scar tissue.

Mallory, White and Salcedo-Salgar (1939) established the criteria for judging the age of infarcts in more detail and these have formed the basis of studies for many workers. In investigating the speed with which healing of myocardial infarction occurs thoroughly described the histologic changes in the lesion and then they correlated these changes with the gross appearance. Their material consisted of seventy-two cases. Evidence as to the age of the infarct was obtained from the history and on clinical grounds. Microscopically, using routine histologic technique, they observed the following:

- Necrosis of muscle fibres, although probably starting immediately as a result of stoppage of the blood supply to a portion of the myocardium, is not observed until six hours had elapsed. The involved muscle fibres begin to appear somewhat hyaline and the striations become somewhat difficult to see. The fibres are swollen and deeply eosinophilic.

- Haemorrhage into infarcts is variable and is present in the form of distended venules and capillaries, but true extravasation around muscle fibres is comparatively rare.

- Infiltration by polymorphnuclear leucocytes occurs during the first twenty-four hours and progressively increases during the first four days, thereafter they gradually disappear.

- Infiltration by eosinophils is marked during the fourth to the eighteenth day.

- About the fifth day, newly formed blood capillaries are seen growing into the infarcted area accompanied by fibroblasts.

- By the tenth day numerous pigmented macrophages are present.

- By the end of the second week muscle fibres are completely removed from small infarcts (3 - 4 mm in diameter), but remain until the third week in larger infarcts.

- Moderate numbers of lymphocytes and plasma cells are found in infarcts as soon as muscle fibres begin to be absorbed.

- Newly formed collagen fibres are found first at about twelve days, become moderately prominent at three weeks and reach their maximum at about two to three months to form a dense contracted scar.

Grossly, the earliest change after infarction noted by Mallory, White and Salcedo-Salgar (1939), was observed during the first forty-eight to seventy-two hours.

- The involved area of myocardium appears paler and dryer than normal. Focal blotchy-red purple areas of haemorrhages are sometimes found. Areas infiltrated by leucocytes appear yellow brown in colour. These changes are slight at first but become progressively more distinct.

- At about the fourth day the periphery of the infarct is marked by a fine yellow border.

- By the sixth to the eighth day, as the leucocytic infiltration becomes more extensive, the yellow band becomes broader in extent and sometimes yellow green in colour.

- After eight to ten days a reddish purple zone is found around the periphery of the infarct due to the formation of granulation tissue.

- The thickness of the myocardium in the infarcted area decreases due to resorption of necrotic fibres, while the peripheral zone of granulation tissue is depressed below the surface.

- With the progress of healing, the infarct becomes pale grey, eventually becomes transformed into a shrunken firm white fibrous scar, thereafter no further changes take place.

Lodge-Patch (1951) also reported on the morphological alterations of myocardial infarction. His material consisted of eighteen autopsy cases, the onset of myocardial infarction was accurately timed in each case. The sequence of the gross and histologic changes observed by this author agrees in general with the observation of Mallory, White and Salcedo-Salgar (1939). However, he was able to draw up a fairly precise timetable of events both macro- and microscopically, especially during the first twenty-four hours. Hence, the morphologic changes occurring during this period only are described. Macroscopically, the earliest change after coronary occlusion was observed at about fifteen hours. The infarct at this stage was described as being usually pale, swollen and oedematous. Microscopically, the earliest sign after myocardial infarction was observed at five hours and involved the muscle nuclei, some of which were noted as swollen and pale and showed a crenated nuclear membrane. At about six to twelve hours, the muscle fibres appeared hyaline and the striations were less prominent. Neutrophil polymorphnuclear leucocytes were the typical cells of the earliest stages and were observed at about six hours. They rapidly increased after twenty-four hours. Oedema was also described as an early feature accompanying the appearance of neutrophils.

It appears from the foregoing studies on the morphologic features of acute myocardial infarction in man that there is a relatively long interval between the moment of damage to the myocardium and the appearance of the first micro- and macromorphologic changes. Light microscopic alterations do not permit any determination as to the time of cell damage before the elapse of a period of five or six hours, while unequivocal macroscopic changes require fifteen to forty-eight hours to become apparent.

Experimental myocardial infarction

The study of the morbid anatomy of experimental myocardial infarction has been widely attempted by many investigators. Early experiments on surgical coronary artery occlusion in dogs by Cohnheim and von Schultess-Rechberg (1881) revealed a change in colour of the portion of the myocardium nourished by the experimentally occluded blood vessel. It became at first pale and soon afterwards changed to a bluish grey colour. More details were reported by Baumgarten (1899) who ligated various branches of the left and right coronary arteries in eight cats and six dogs for the purpose of mapping out the distribution of the coronary arteries by means of the infarctions which followed the ligation. The more recent infarct examined in cats and dogs were thirty hours and twenty-two hours respectively. Macroscopically, the infarcted area appeared white or whitish yellow in colour, opaque and flaccid. In only three cases the infarct was described as haemorrhagic. Microscopically, coagulation necrosis was fully developed as early as twenty-two hours. At this time many nuclei lost their affinity for stains, some showed irregularities and fragmentation and a few were swollen and vacuolated.

In 1916, Karsner and Dwyer published the results of a thorough investigation on the gross and histologic alterations in experimental myocardial infarction in its various stages, taking the time element for healing into consideration. The age of the earliest infarct, produced in thirteen dogs by ligating the anterior descending branch of the left coronary artery, was one-half hour and the oldest was seventy days. Immediately after ligation they noticed an area of

distinct purple discolouration, which was then replaced by pallor in the one-half hour infarct and at two days the infarct became gradually more sharply defined. They summarised their observation by stating that grossly the myocardial infarct was primarily an area of congestion which has later undergone coagulation necrosis, the area gradually showed decolourisation, became smaller, more sharply defined, removed from the site of arterial occlusion, and in the later stage was accompanied by thinning of the heart wall. Histologically, congestion, interstitial oedema and haemorrhages were the earliest manifestations of the lesion and appeared in the one-half hour infarct accompanied by slight diminution of the transverse striations of the muscle fibres. Hyaline necrosis, nuclear changes and polymorphnuclear leucocytic infiltration appeared at the end of twelve hours. After twenty-four hours numerous fibroblasts were seen about the blood vessels and under the endocardium, with progressing age and after eighteen days the infarct was present as a well defined scar.

Similar histologic changes in experimental ligation of the left anterior descending coronary artery (with its accompanying veins) in dogs have been reported by Tennant, Grayzel, Sutherland and Stringer (1936), and by Bronson (1938), in short-term coronary artery occlusion. Blumgart, Gilligan and Shlesinger (1941), in investigating the period of ischaemia required to produce myocardial infarction in dogs by temporary ligation of a coronary artery, observed that the myocardium appeared grossly cyanotic immediately after ligation. They reported that the time relations between the various microscopic changes and the interval after operation agreed closely with those observed by

Mallory, White and Salcedo-Salgar (1939) in their study of human myocardial infarction.

Recent experimental studies on early myocardial infarcts in dogs by Shnitka and Nachlas (1963), using light microscopy and specific stains, revealed mitochondrial swelling as first evidence after four hours of coronary ligation, while fragmentation of myofibrils first appeared in infarcts of six hours' duration. They stated that, although the rapidity of appearance of myocardial changes in different mammalian species appears to vary inversely with the size of the heart, the early histologic alterations observed in the dog were similar to those observed by Mallory, White and Salcedo-Salgar (1939) in man.

Experimental ligation of the left circumflex coronary artery in dog followed by release of the ligature and recirculation of the affected part is reported to increase the rate of onset of the infarction process (Sommers and Jennings, 1964). Hence, the precise order and speed of events may be locally influenced by blood flow at the periphery of the lesion.

Myocardial enzymes in myocardial infarction

The myocardium, as all other tissues, contains an abundant concentration of many enzymes. It is believed that myocardial tissue, once metabolically damaged, releases its content of enzymes into the interstitial fluid and then into the blood. If an area of heart muscle becomes ischaemic and necrotic, the concentration of enzymes in that area, therefore,decreases. This assumption was proved by biochemical investigations and experimental studies which have demonstrated that infarcted muscle is rapidly depleted of various enzymes which appear in an active form in the blood that drains from the infarcted myocardium. LaDue, Wróblewski and Karmen (1954) were the first investigators to report that the serum level of glutamic-oxaloacetic transaminase (GOT), now known as aspartate transaminase (AST) (Gray and Howorth, 1977), which is most concentrated in heart muscle (Cohen and Hekhuis, 1941) and which accounts for approximately 1.6% of the dry weight of the heart (Green, Leloir and Nocito, 1945), is elevated within twelve to twenty-four hours after an acute myocardial infarction to levels two- to twenty-fold normal, gradually falling off to normal within three to six days. Experimental evidence was supplied by Nydick, Wróblewski and LaDue (1955) who produced myocardial infarction in dogs by coronary arteries ligation. They observed an increase in the serum glutamic-oxaloacetic transaminase activity which occurred within six hours following ligation. They further reported that both the degree and duration of enzyme abnormality were proportional to the size of the infarct, and suggested that the mechanism of elevation

of transaminase activity was through release of the enzyme into the blood stream following an increase in the permeability of the injured heart muscle cell.

Graves, Koepke, LaFond and Ross (1962) analysed the concentration of glutamic-oxaloacetic transaminase in heart homogenates of forty patients, obtained at necropsy. One of their purposes was an attempt to determine if the decrease in the enzyme concentration can be demonstrated before the morphologic changes of acute myocardial infarcts are recognizable. Significant decrease in myocardial transaminase without anatomic changes was demonstrated in three cases of presumed early infarcts. As the group of patients was small, they stated the results are indicative but not conclusive.

A search for other enzymes in heart muscle which may behave similarly during myocardial infarction was made by Wróblewski and LaDue (1955), who noticed the presence of lactate dehydrogenase (LDH) in serum. This observation was followed by the estimation of the enzyme activity in dog tissue homogenates (Wróblewski, Ruegsegger and LaDue, 1956). The high content of lactate dehydrogenase activity in cardiac muscle and relatively low serum values suggested the possible usefulness of studying its serum levels in myocardial infarction. A rise in the serum levels of lactate dehydrogenase within twelve to twenty-four hours in experimental and human acute myocardial infarcts was also noted by the latter group of workers. Increased levels occurred even in the absence of definitive electrocardiographic changes (MacDonald, Simpson and Nossal, 1957).

Corresponding biochemical assays of myocardial infarction were reported by Jennings, Kaltenbach and Smetters (1957). These authors studied the pattern of loss of glutamic-oxaloacetic transaminase, lactate and succinate dehydrogenases from homogenates of myocardial fibres injured irreversibly by ischaemia in a homogeneous infarct in dogs, induced by ligation of the left circumflex coronary artery near its origin (Jennings, Wartman and Zudyk, 1957). After an initial period of little or no decrease in enzyme activity, lasting forty to seventy minutes with glutamic-oxaloacetic transaminase, two hours with lactate dehydrogenase, and four to five hours with succinate dehydrogenase, the tissue levels of all three enzymes rapidly decreased until levels of thirty to fifty per cent of normal were reached twelve to fifteen hours after ligation. They postulated that there must be defects of sufficient size in the semipermeable cell membrane to allow the enzyme to leak out, and the difference in the time interval before the enzyme can begin to leak into the extracellular fluid seemed to depend on the molecular weight of the enzyme, and the increasing damage to the cell membrane with time.

Ruegsegger, Nydick, Freiman and LaDue (1959) provided evidence about leakage of enzymes from the infarcted areas into the blood stream. In estimating the serum activity patterns of glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase and lactate dehydrogenase in experimental myocardial infarction in dogs, they observed that the most rapid rise in serum enzyme concentration corresponded to the period during which there was the most precipitous decrease in concentration

in the infarcted myocardium. They also noted that the enzyme concentration in the infarcted muscle diminished progressively with the duration of the infarction, and that following myocardial infarction, coronary sinus blood was consistently ten to fifteen per cent richer in each enzyme studied than was peripheral blood obtained simultaneously. Lastly, they observed that the relative rises of serum enzyme activity were proportional to the original concentration gradients between myocardium and the serum for each enzyme, as well as the size of the infarct. These observations were confirmed the same year by Strandjord, Thomas and White (1959),who investigated the enzyme activity of iso-citrate and lactate dehydrogenase in the homogenates prepared from normal and experimentally infarcted dog myocardium. In addition to similar evidence provided by Ruegsegger, Nydick, Freiman and LaDue (1959) they noted that when the coronary sinus activity was no longer greater than the activity found in the vena cava, the coronary sinus and vena cava enzyme activities had both returned to normal, suggesting that the observed elevations were primarily a direct result of enzyme release from the myocardium.

The observation of normal values of serum lactate dehydrogenase in patients with apparent myocardial infarction and elevated values in other disease states, lent emphasis to the basic investigation of the nature and mechanism of action of lactate dehydrogenase. Kaplan, Clotti, Hamolsky and Bieber (1960), in a series of studies to measure lactate dehydrogenase activities of different tissues in several vertebrate and invertebrate species, used electrophoretic and immunologic

techniques, to show differences in lactate dehydrogenase of different species, and in different tissues of the same individual organism. Their results suggested the possibility of defining more precisely the tissue of origin of the elevated serum levels. Wróblewski, Gregory and Ross (1960), and Wróblewski and Gregory (1961) described a characteristic electrophoretic pattern of five "isozymes" of lactate dehydrogenase (LD1 to LD5) in normal subjects. In fifteen patients with acute myocardial infarction, an elevation of one component was observed and considered more specific for myocardial damage than total lactate dehydrogenase activity. They further reported that elevation of this component was observed in certain cases with small or sub-endocardial infarcts at times when total plasma lactate dehydrogenase was within the normal range. This was confirmed by Preston, Briere and Batsakis (1965) who demonstrated that tissues with a high aerobic metabolism, such as cardiac muscle, have a high content of fast-migrating (anodic) isoenzymes. Conversely, slow-migrating (cathodic) fractions predominate in the liver. The various forms of lactate dehydrogenase are made up of four subunits of two parent molecules which are designated H and M. The pure H type, occurring mainly in heart muscle, is composed of 4 H subunits (H_4) while the pure M type from skeletal muscle consists of 4 M subunits (M_4). Three other molecular hybrids also exist.

Elliot and Wilkinson (1961) have shown that lactate dehydrogenase does not possess an absolute specificity for its usual substrate (L-lactate), but can readily remove hydrogen from hydroxybutyrate

substrate. They further observed that the fast-migrating serum lactate dehydrogenase isoenzymes exhibit much greater ability to reduce α-ketobutyrate than do the slow fractions. The use of hydroxybutyrate dehydrogenase (HBD) determination to measure the contribution of the cardiac fraction to the total lactate dehydrogenase was described by Elliot, Jepson and Wilkinson (1962) and by Coodley (1966, 1968) who indicated the usefulness of this enzyme determination in the diagnosis of myocardial infarction, since it reflects the identification of that fraction of lactate dehydrogenase specifically related to myocardial infarction. Serum lactate dehydrogenase isoenzyme fractions appeared to have a superior sensitivity. The appearance of a positive increase in the fast-migrating fractions may antedate both electrocardiographic changes and a rise in the total enzyme activity in the serum (Freeman and Opher, 1965; Batsakis and Briere, 1966). It has also proved to be valuable in the detection of acute myocardial infarction postoperatively (Mohiuddin, Raffetto, Sketch, Lynch, Schultz and Runco, 1976; Codd, Sullivan, Weins, Barner, Kaiser and Willman, 1977).

The activity of myocardial malate dehydrogenase was, likewise, investigated in cases of myocardial infarction. Serum levels were comparably increased in both experimental and clinical myocardial infarction (Wacker, Ulmer and Vallee, 1956; Siegal and Bing, 1956; Bing, Castellanos and Siegal, 1957; Rodney and Shapiro, 1959; Aquilina and Farnsworth, 1960). It would appear, however, that its enzymatic assays have not withstood critical clinical and laboratory investigations.

Other myocardial enzyme activities in myocardial infarction which may reflect an enzyme leakage that occurs from the infarcted area into the circulation have been investigated. It was observed that creatine phosphokinase (CPK) was the first to respond, with a rise in the serum level within three to six hours of infarction (Hess, MacDonald, Frederick, Jones, Neely and Gross, 1964; Smith, 1967). Three isoenzymes of creatine phosphokinase were separated electrophoretically. The fast-migrating (BB) is of cerebral origin, while the slowest is of muscle origin (MM). The third isoenzyme of intermediate mobility (MB) is the cardiac specific fraction, which accounts for twenty to thirty per cent of the total creatine phosphokinase plasma activity after acute myocardial infarction (Welman,Selwyn, Peters, Colbeck and Fox, 1978). Myocardial creatine phosphokinase depletion was reported to reflect infarct size under experimental conditions, and its release into the circulation has been correlated with histologically demonstrable necrosis (Kjekshus, 1976), and was successfully used for the detection of postoperative acute myocardial infarction (Warren, Wagner, Bethea, Roe, Oldham and Kong, 1977). However, the electrocardiographic changes in patients with acute myocardial fraction, indicating loss of electrically active myocardium, were almost complete before the cardio-specific creatine kinase appeared in the plasma (Selwyn, Fox, Welman, Jonathan and Shillingford, 1978). Recently it has been reported that the extent of myocardial injury in man can be assessed quantitatively using the calculated amounts of enzyme released, as well as using peak plasma activities of creatine kinase cardiac fraction and hydroxybutyrate dehydrogenase (Van Der Laarse, Davids, Hollaar,Van Der Valk, Witteveen and Hermens, 1979).

The latest contribution to the clinical diagnosis of myocardial infarction is the reported rise in the serum level of the respiratory pigment, myoglobin, in patients with acute myocardial infarction (Stone, Waterman, Harimoto, Murray, Wilson, Platt, Blomoqvist and Willerson, 1977). Using radioimmunoassay, transient myoglobinaemia was detected. The myoglobin levels were about ten times on average higher than those found in the sera of normal persons, and the raised myoglobin levels preceeded the rise in creatine kinase values. This finding was based on the observation that myoglobinuria was transiently detected in some patients with acute myocardial infarction (Kessler, Liebson, Mattenheimer and Adams, 1975), and on the suggestion that damage to the cardiac muscle might result in release of myoglobin into the circulation, the same as with myocardial enzymes. Substantial evidence was provided in experimentally-induced acute myocardial infarction in dogs, which showed that serum myoglobin levels begin to rise within two hours and peak in six hours (Willerson, Poliner, Buja, Waterman, Gomez-Sanchez, Templeton and Stone, 1976). These authors further reported that the peak serum myoglobin levels correlated with infarct size determined histologically.

Myocardial enzyme histochemistry in early infarction

With the knowledge of increased serum levels of several enzymes shortly after myocardial infarction and the concurrent demonstration of decreased activities of these enzymes in the infarcted area of the heart, enzyme histochemistry was introduced in the hope that it would then be possible to diagnose an early ischaemic injury to the myocardium. Conventional histologic methods fail to reveal the existence of myocardial infarction in its early stages, and enzymatic techniques could redress the balance.

Microenzymatic reactions

Heart muscle cells are characterised by the highest activity of oxidoreductive enzymes. Seligman and Rutenburg (1951) were among the first to report on the histochemical staining pattern of succinate dehydrogenase in mammalian organs. Rutenburg, Wolman and Seligman (1953) studied the distribution of this enzyme in different organs in various laboratory species and found that the heart contained the largest amount of stainable enzymatic activity. The myocardium of the human heart, likewise, revealed large amounts of stainable succinate dehydrogenase. The abundance of this enzyme in heart muscle indicated its fundamental importance in the metabolic processes that take place during cardiac contraction, and it has long been recognised that succinate dehydrogenase is one of the important enzymes in biologic oxidation.

Bourne and Malaty (1953) observed a marked decrease of microscopically demonstrable succinate dehydrogenase activity in the heart as well as in other organs of rats following bilateral adrenalectomy. Since the reported findings in the heart were similar to those in myocardial necrosis, Wachtein and Meisel (1955) were led to study the distribution of this enzyme in human myocardial infarction and in experimentally induced myocardial infarction in rats. Using frozen sections, reduction of enzyme activity was noticed as early as one and a half to two hours after the onset of acute symptoms in many muscle fibres that showed no unequivocal microscopic evidence of damage with routine stains. They also noticed that in some of these fibres, the reduced tetrazolium (formazan) was not deposited in the usual granular form but rather in irregularly shaped, larger particles. Cross striations appeared entirely normal in many muscle fibres with reduced deposition of the dye. In myocardial infarction in rats induced by intraperitoneal injections of fluoroacetate and plasmocid, dehydrogenase activity in damaged fibres disappeared rapidly or was markedly reduced. They recommended the use of the histochemical staining reaction of succinate dehydrogenase for the evaluation of myocardial damage under experimental conditions and in necropsy material.

Burstone and Miller (1961) examined cytochrome oxidase activity in a series of ten human myocardial infarctions, using one of the newer histochemical techniques for this enzyme. A decline in mitochondrial cytochrome oxidase activity was observed within thirty-

six hours after coronary occlusion. Monis and Weinberg (1964) investigated aminopeptidase activity in human myocardial infarction. Heart tissue obtained from thirty-six necropsies of myocardial infarction in its various stages and eight control cases were studied. They recorded that the normal myocardial fibres contained no histochemically demonstrable aminopeptidase activity. In the normal heart the enzyme was confined to mast cells. The aminopeptidase that appeared during infarction was at first located in the polymorphnuclear leucocytes which begin to infiltrate the lesion about twelve to twenty-four hours after coronary occlusion. The oedematous interstitial fluid which appears at an early stage between injured heart muscle fibres also exhibited abundant aminopeptidase activity. Later, during organization of the infarct, this enzyme activity appeared within the proliferating connective tissue cells, macrophages and fibroblasts, but subsequently disappeared as the tissue was scarred. Presumably these aminopeptidases were concerned with protein degradation in necrotic muscle fibres and with the synthesis of new protein for collagen fibres (Adams, 1967).

Morales and Fine (1966) carried out a very detailed investigation on the histochemical characteristics of early human myocardial infarction, using eleven hearts obtained at necropsy from clinically suspected cases of myocardial infarction. They observed that myocardial fibres did not show any microreactions for acid phosphatase, glucose 6-phosphatase, leucine aminopeptidase, dihydroxy-phenylalanine oxidase, peroxidase, alkaline phosphatase, and 5-nucleotidase. Very weak or

negative reactions were obtained with glutamate, alcohol, glycerophosphate, 6-phosphogluconate, and glucose-6-phosphate dehydrogenases. Phosphorylase activity was absent in postmortem myocardium, but was strong in that obtained from two open heart operations. Strong reactions were obtained for non-specific esterases. The moderate activity and irregular distribution of adenosine triphosphatase in heart muscle did not permit easy recognition of its depletion. The most striking early change they observed was depletion of oxidative enzymes: glutaminase I, B-hydroxybutyrate and malate dehydrogeanses, NADPH (reduced nicotinamide adenine dinucleotide phosphate) and NADH (reduced nicotinamide adenine dinucleotide) diaphorases, lactate and succinate dehydrogenases, and cytochrome oxidase in that order, and were the most consistent and useful enzymes depicting early myocardial damage. They concluded that decreased enzyme activity was a better and more easily recognised indicator of early human myocardial damage than conventional histologic and non-enzyme histochemical methods. Loss of B-hydroxybutyrate and glutaminase I were the most sensitive and valuable indicators of early human myocardial infarction. Depletion prior to one and a half hours after the onset of infarction was only observed with these two enzymes, being confined to scattered myocardial fibres, and became more pronounced and diffuse with increased duration of infarction. Malate dehydrogenase depletion was demonstrated by Knight (1967), who noticed a microscopic decrease in its activity in suspected infarcted areas of human myocardium as early as two to three hours after the onset of ischaemia.

Macroenzymatic histochemistry

The forementioned histoenzymatic reactions for recognizing myocardial infarction are clearly of value in the histological diagnosis of myocardial infarcts, for such changes are apparent before structural alterations can be convincingly demonstrated with conventional staining methods. However, if the infarct cannot be seen and identified with the naked eye at autopsy, one or two random blocks might well fail to detect its presence.

Neoral, Kolin, Kodousek and Kvasnicka (1956, 1959) introduced their original enzymatic macroreaction, the so-called dehydrogenase reaction, for the gross identification of early myocardial infarction. The principle of the reaction is an enzymatic reduction of potassium or sodium tellurite (an oxidation-reduction indicator) to black tellurium mostly by the action of malate dehydrogenase. Normal heart muscle, rich in malate dehydrogenase, stains black. In contrast, infarcted muscle, depleted of enzyme, does not appreciably change colour. The results of their studies on the application of this method to transverse heart slices of experimental myocardial infarction in fifteen dogs, revealed ischaemic areas in the myocardium as early as five hours after coronary ligation.

Kolin, Neoral and Kodousek (1959) applied the tellurite reaction to transverse heart slices of ten patients with suspected myocardial infarction. The earliest infarct which they recognised macroscopically, was in a patient who died seven hours after the onset of clinical symptoms. In 1960, Kolin and Neoral used the same method and published

the results of their studies on macroscopic identification of human myocardial infarcts at necropsy, including ten more patients with suspected or proved myocardial infarction. Recent ischaemic changes of five hours duration after coronary occlusion was detectable, but there were neither gross nor microscopic conventional evidence.

Sandritter and Jestädt (1957/1958) recommended the application of the triphenyl tetrazolium reaction (TTC reaction) to facilitate the gross recognition of inapparent myocardial infarcts at necropsy. The TTC reaction is an enzymatic macroreaction in which triphenyl tetrazolium chloride, used as a hydrogen acceptor, is reduced by the activity of the dehydrogenase system present in normal cardiac muscle to a formazan pigment, which stains normal heart muscle bright brick red. A negative TTC reaction indicated the presence of an infarct. Jestädt and Sandritter (1959) applied the TTC reaction to hearts obtained at necropsy from fifty-eight clinically diganosed cases of myocardial infarction, forty control cases and fourteen cases with pathological evidence of fatty degeneration. The TTC reaction was negative in all the clinically diagnosed cases of myocardial infarcts. In eleven cases there was neither macroscopic nor microscopic evidence of infarction and the TTC reaction was negative as early as three to four hours after the onset of clinical symptoms. Although the reaction was also negative in the fourteen cases of fatty degeneration, and in five of the forty control cases, they recommended its applicability to reveal the macroscopic presence of early myocardial infarcts and stated that similar results were obtained in experimental infarcts in rabbits.

The TTC reaction was also employed by Knight (1967) as a direct screening technique for human myocardial infarcts at autopsy. Infarcted areas of the myocardium, recognized by complete absence of the dye or a very much reduced deposition, were demonstrated in transverse heart slices as early as five hours after the onset of infarction.

Cain and Assmann(1960) reported fourteen human hearts stained at necropsy with neotetrazolium chloride, which is a redox indicator that stains normal heart muscle bluish black. In six cases there was a clinical diagnosis of myocardial infarction of twenty-four to a few days duration. The method was described as accurately outlining areas of acute myocardial infarction, postinfarction scar and interstitial fibrosis.

Nachlas and Shnitka (1963) confirmed and extended the findings of these workers for the gross identiffcation of early myocardial infarction. They modified the dehydrogenase system, using a different hydrogen acceptor, nitroblue tetrazolium (NBT). Early myocardial infarcts are outlined by differential staining of the myocardium. Reduction of nitroblue tetrazolium to blue formazan by the combined action of endogenous substrate, coenzyme and dehydrogenase present in normal heart muscle reveals viable fibres in dark blue, while infarcted muscle depleted of these substances remains unstained or only faintly stained pink. The results of their studies,using the NBT method, on experimental myocardial infarction in forty dogs demonstrated the area of infarction in transverse heart slices as early as two hours after coronary artery ligation. Their subsequent experience with NBT in slices of twenty-

three human hearts obtained at necropsy from patients with clinical histories of ischaemic heart diseases, revealed an infarct as early as eight hours after the onset of clinical symptoms.

A similar finding was reported by Ramkissoon (1966) who surveyed thirty-one human hearts obtained from patients with clinical histories of arteriosclerotic heart diseases. The earliest myocardial infarction outlined macroscopically by the NBT method was in a patient whose symptoms had begun eight hours before death.

Brody, Belding, Belding and Feldman (1967) examined the hearts of thirty-one patients who died with suspected or proved myocardial infarction. The earliest infarct that was demonstrated with NBT was eighteen hours old, as revealed by the clinical history.

McVie (1970) was able to identify an infarct as early as three and a half hours using the same NBT macroscopic screening test. In a series of twenty cases of sudden death within twelve hours of the onset of symptoms suggesting myocardial infarction, the NBT method revealed infarcts in ten of the twenty cases which were not seen to be infarcted on histological grounds.

Kalderon (1968) also found the NBT method most useful in cases of sudden death. Out of twenty-six cases examined, seven revealed positive diagnostic results, five of which were cases of sudden death within twelve hours from the onset of symptoms.

Anderson and Hansen (1973) estimated the frequency with which fresh myocardial infarctions escape recognition at autopsy by using

the NBT reaction on the hearts in one hundred consecutive autopsies. The gross staining demonstrated the presence of fifteen cases of fresh myocardial infarction where ordinary macroscopic evaluation had given no suspicion of fresh infarction. Presumably the age of these infarcts was under twenty-four hours.

The foregoing discussion reflects the usefulness of the dehydrogenase macroreaction for the identification of early inapparent myocardial infarcts at necropsy. Nitroblue tetrazolium is the favoured oxidation reduction indicator. Yet, it still remains unclear in man how soon after infarction the NBT enzymatic macroreaction becomes diagnostic.

Aim of the work

The search for a reliable procedure to diagnose early human myocardial infarction at necropsy, when the time interval is too short for the classical changes to have developed, has been of great concern to pathologists. Conventional autopsy of the heart fails to demonstrate myocardial infarction in its early stages.

The demonstration of early ischaemic changes in the myocardium is important in several ways: -

First, as a confirmation that ischaemic heart disease really is the cause of death. In the absence of an apparent myocardial lesion and in the presence of a diseased coronary artery, the cause of death is often listed as a coronary artery disease. This is a diagnosis of exclusion which has prevented the incrimination of the myocardial lesion as the immediate cause of death in some patients whose symptoms have been of short duration.

Second, ischaemia of the myocardium may kill suddenly. If death is rapid, an infarct could never be detected at necropsy. Its presence can be presumed from circumstantial evidence only. Even the finding of a fresh thrombus occluding a coronary artery is not a proof of subsequent infarction. Here it is possible that death resulted from ventricular arrhythmia.

Third, from a medicolegal point of view, the demonstration of early myocardial infarct may help to explain traffic and other types of accident, where there is reason to suspect that the victim may have suffered sudden cardiac failure.

Fourth, a better understanding of the early ischaemic changes in myocardial muscle is essential if a more effective therapy is to be devised for acute myocardial infarction.

Lastly, a consistent and reliable macroscopic procedure that can demonstrate an infarct at autopsy would overcome the delay involved in waiting for the microscopy.

For such practical reasons,the dehydrogenase macroreaction was introduced. Because of the uncertainty about how soon after infarction the enzymatic macroreaction can be diagnostic, the present study was carried out with the following aims:-

1. Study of the applicability of macroscopic enzyme histochemistry for the gross identification of early human myocardial infarction at necropsy. A modified nitroblue tetrazolium (NBT) dehydrogenase macroreaction was applied (Derias and Adams, 1978). The method is compared with Nachlas and Shnitka's (1963) original nitroblue tetrazolium method, which has been adopted by all workers who are interested in this particular field of study.

2. The correlation of the age of the infarct, as indicated by the clinical history or the clinical and laboratory diagnosis, or both, with the gross histochemical appearance of the myocardium, and to determine how soon after infarction the method can be diagnostic in man.

3. To correlate the gross histochemical appearance of the myocardium with the subsequent histological findings in the damaged myocardium, particularly to assess the validity of the NBT method.

4. The evaluation of the conditions that provide the maximal NBT enzymatic macroreaction in the normal and in the damaged myocardium:

   a. The assessment of the role of coenzyme NAD (nicotinamide adenine dinucleotide) and of the respiratory chain inhibitor, cyanide, in the dehydrogenase macroreaction.

   b. The effect of postmortem autolysis on the macroreaction, viz., death-necropsy interval, storage of hearts at 4^{o} or -17^{o} or ambient temperature.

   c. Estimation of the period of effective staining with endogenous substrate in regard to the death-necropsy interval.

   d. The effect on the macroreaction of including an electron transfer mediator (phenazine methosulphate) in the NBT incubating medium.

5. The effect of the subject's age on the macroscopic enzyme reaction.

6. Enzymes other than the dehydrogenases were tested macroscopically for the first time to investigate their possible use in the gross identification of early human myocardial infarcts at autopsy. This attempt, to the worker's knowledge, had not previously been tried in the macroscopic study of human or experimental myocardial infarction. These enzymes were :-

Glutamic-oxaloacetic transaminase, myoglobin peroxidase, monoamine oxidase, creatine phosphokinase (this enzyme was recently examined macroscopically in experimental myocardial infarction in dogs by Anderson, Popple, Parker, Sayer, Trickey

and Davies, 1979), phosphorylase, non-specific esterase, acid phosphatase, and aminopeptidase.

7. The application of a non-enzyme histochemical method - Phloxine tartrazine (Lendrum, 1947) as an aid in the microscopic identification of early human myocardial infarction in tissue sections. Conventional non-enzyme histochemical methods were also assessed as to their value as microscopic aids.

8. Assessment of the nature and the reliability of the wavy myocardial fibre as a morphologic index of early myocardial infarction.

CHAPTER II

MATERIALS

One hundred and seventy-nine hearts were obtained at necropsy (Table I).

Table I

Source of the human hearts obtained at necropsy

Source of material	Number of hearts
Hospital cases	64
Forensic cases	115
Total	179

Sixty-four cases were hospital patients and one hundred and fifteen cases were coroner's postmortems. The hospital autopsies were examined by the pathologist on duty from the Department of Histopathology. The forensic cases were examined by a pathologist from the Department of Forensic Medicine. The necropsies were performed from two hours to five days after death (Table 2).

These hearts provided the material examined for the gross enzymatic identification of recent myocardial infarct (one hundred and eight hearts). Fifty-one hearts served as controls, and thirty-three hearts were tested for the effect of postmortem autolysis on the enzymatic macroreaction. A further miscellaneous group of ten hearts were also obtained. Table 3 illustrates the distribution of the material examined.

Table 2: The death-necropsy Interval of the one hundred and seventy-nine cases.

Hours after death	Number of cases
Two	3
Three	4
Four	2
Five	5
Six	2
Seven	3
Nine	1
Ten	1
Twelve	2
Sixteen	1
Eighteen	2
Twenty	4
Twenty one	2
Twenty two	9
Twenty four	68
Twenty seven	4
Thirty two	1
Thirty six	3
Forty eight	34
Seventy two	21
Ninety six	6
One hundred and twenty	1

Table 3: Classification of the hearts examined

Distribution of material	Number of hearts
Macroenzymatic identification of recent myocardial infarcts	
- Hospital	28
- Forensic	80
Controls	51
Postmortem autolysis*	33
Miscellaneous group	10

*Twenty three hearts included in this group were provided from the control cases.

Macroenzymatic identification of recent myocardial infarction

For the purpose of identifying early myocardial infarcts, one hundred and eight hearts were examined and these included both hospital (twenty-eight hearts) and forensic cases (eighty hearts).

The hearts obtained from hospital cases were from patients who were admitted because their histories were clinically suggestive of recent or impending myocardial infarction, or when the diagnosis was established on clinical grounds (laboratory and electrocardiographic changes). Also included were hearts from patients who died unexpectedly without previous evidence of coronary artery disease, and from patients who died in the intensive care unit. These clinical data were obtained from the medical records of the patients. Table 4 shows the number of cases falling into each of these clinical categories.

The hearts in the forensic cases were obtained as follows:- from cases of sudden death; from subjects who died on admission to the hospital after collapse at home, at work, or in the street; from subjects found dead in bed; and from patients who had died in the operating theatre (Table 5).

The clinical age of the infarct is defined here as the estimated time between the onset of acute myocardial infarction and death. The suspected cases of recent myocardial infarction were all taken from patients with a clinical age of less than 24 hours. Forty-eight cases were estimated to be of clinical age under one hour; twenty-eight cases

Table 4. The clinical categories of the hospital patients from whom the hearts were obtained at necropsy

Types of hospital patients	Number of cases
Ischaemic heart disease:	
- History suggestive of recent myocardial infarct	13
- Laboratory and electro-cardiographic evidence	5*
Died unexpectedly. No previous evidence of coronary artery disease	8
Intensive care unit	2
Total	28

* In three cases the ECG showed evidence of ischaemic changes only.

Table 5: Distribution of the forensic cases according to the mode of death

Mode of death	Number of cases
Sudden death	21
Died on admission to hospital after collapse:	
- at home	16
- in the street	17
- at work	14
Found dead in bed	9
Died in the operating theatre	3
Total	80

of clinical age of one to five hours; twenty-one cases of five to twelve hours, and eleven cases of twelve to twenty-four hours (Table 6).

In some cases it was difficult to compute accurately the clinical age of the infarct, and such cases were included in the nearest appropriate clinical age group. For example, some patients died on admission to the hospital without providing a history after collapse at home, in the street or at work. It was difficult with these to estimate the exact clinical age of the suspected infarct, so they were included in the clinical age of one to five hours. Also subjects who were found dead in bed on the next day were grouped under the clinical age of five to twelve hours. Cases with a longer and often obscure history stretching back to the previous day were assigned a clinical age of twelve to twenty-four hours.

Controls

Fifty-one control hearts were examined in this study (Table 7). They were obtained at necropsy from randomly selected cases. They comprised:-

- negative controls (thirty-seven hearts): these were from subjects free of heart disease. In none of these cases was acute myocardial infarction suspected clinically or confirmed pathologically. Table 8 shows the cause of death in this group.

- positive controls (fourteen hearts) : a group of clinically proven myocardial infarctions and confirmed both at autopsy and histologically. The clinical age varied from forty-eight hours to ten days.

Table 6: The clinical age of the one hundred and eight suspected cases of recent myocardial infarction

Clinical age	Number of cases		Total number
	Forensic	Hospital	
Under 1 hour	40	8	48
1 - 5 hours	22	6	28
5 - 12 hours	14	7	21
12 - 24 hours	4	7	11
Total	80	28	108

Table 7: Types of controls examined

Control	Number of hearts
Negative control	37
Positive control	14
Total	51

Table 8: The cause of death in the negative controls

Cause of death	Number
Road traffic accident	13
Malignant disease	10
Bronchopneumonia	8
Chronic renal failure	3
Homicide	2
Train accident	1

Postmortem autolysis

Thirty-three hearts were examined for the effect of postmortem autolysis on the macroenzymatic reaction. The shortest death-necropsy interval was two hours and the longest was one hundred and twenty hours (Tables 9 and 10).

- Three normal hearts were kept at an ambient temperature between 18^{o} and 25^{o} for periods of twenty-four to seventy-two hours, and seven normal hearts were kept at 4^{o} for seven days. They were sampled at intervals of twenty-four hours.
- Three normal hearts were kept at 4^{o} for fourteen days, and two normal hearts were kept at -17^{o} for fourteen days. These were sampled at the seventh and at the fourteenth day.
- Four hearts, where the enzymatic macroreaction revealed a recent myocardial infarct, were kept at 4^{o} and at -17^{o} for fourteen days, and were sampled at the seventh and at the fourteenth day.

In all these cases the result of the macroenzymatic staining was compared with the freshly stained heart slice from each case.

Fourteen normal hearts were used to study the effect of postmortem autolysis (the death - necropsy interval) on the gross enzymatic reaction. The death - necropsy interval was one hundred and twenty hours in one case; ninety-six hours in three cases; seventy-two hours in three cases; forty-eight hours in three cases, and twenty-four hours in four cases.

Table 9: The time allowed for postmortem autolysis at various temperatures

Time allowed for autolysis	Temperature	Number of hearts
Seventy two hours	18 - 25°	3 (normal hearts)
Seven days	4°	7 (normal hearts)
Fourteen days	4°	3 (normal hearts)
Fourteen days	-17°	2 (normal hearts)
Fourteen days	4°	2 (recent M.I.*)
Fourteen days	-17°	2 (recent M.I.)
Total		19

* M.I. = Myocardial infarction

Table 10: The number of normal hearts examined for postmortem autolysis in relation to death-necropsy interval

Death-necropsy interval (hours)	Number of hearts
120	1
96	3
72	3
48	3
24	4
Total	14

Miscellaenous Group

This group consisted of ten hearts obtained from patients who died of illness other than myocardial infarction, but in whom it was thought that hypoxia and shock might have caused damage to the myocardium. In none of these cases was acute myocardial infarction suspected clinically. At autopsy the coronary arteries were not significantly diseased and none of them showed occlusive thromboses. The clinical diagnosis of these cases is demonstrated in Table 11.

The effect of ageing on the macroenzymatic reaction

Of the one hundred and seventy nine cases examined, one hundred and thirty one (73.2%) were males and forty eight (26.8%) were females. The mean age at the time of death was 61 years for the females and 54 years for the males. With the material used for studying the effect of age on the macroenzymatic reaction, the youngest case was six weeks old and the oldest was eighty-seven years old. Table 12 shows the age and sex of the cases examined.

Examination of the hearts

After sectioning of the coronary arteries at 0.5 cm intervals as far as their main and primary sub-branches were visible, the heart was sliced transversely at approximately 1 cm intervals from the apex to the base through both ventricles. Each heart slice was inspected carefully for

Table 11: The clinical diagnosis of the miscellaneous group

Serial number	Clinical diagnosis
1	Severe megaloblastic anaemia
2	Congestive cardiomyopathy
3	Rheumatic mitral stenosis
4	Calcific aortic stenosis
5	Carcinoma of the stomach
6	Subarachnoid haemorrhage
7	Coronary artery hypoplasia
8	Acute corrosive poisoning
9	Chest infection
10	Ellis-Van Creveld syndrome

Table 12: Age and sex distribution of the one hundred and seventy-nine cases

Age (decades)	Number	
	Male	Female
6 weeks		1
3 months		1
5 years	1	
8 years		1
9 years		1
10-20	4	1
21-30	3	1
31-40	3	1
41-50	20	6
51-60	32	5
61-70	44	11
71-80	21	13
81-90	3	6
Total	131 (73.2%)	48 (26.8%)

any macroscopic evidence of myocardial damage. The heart slices were rinsed in cold running tap water to remove traces of blood before they were incubated in the various media employed for the macroenzymatic study as set out below.

Two to three blocks were taken for histology from each heart and, in infarction cases, one block was taken from the mirror-image surface and one or two blocks from the adjacent normal myocardium. The blocks were fixed in 10% neutral formol saline and embedded in paraffin wax. They were all examined microscopically, using sections stained with haematoxylin-eosin (H.E.) or selected histochemical techniques. Diseased coronary arteries were decalcified with 7% EDTA (ethylenediaminetetra-acetic acid), taken to paraffin and stained with H.E.

CHAPTER III

METHODS

Enzyme and non-enzyme histochemical methods were applied in the present study for the respective macroscopic and microscopic identification of early human myocardial infarcts.

Enzyme histochemical methods

Histochemical methods were tested for their capacity to demonstrate the activities of the following enzymes in gross specimens of myocardium, and for their value in delineating foci of infarction.

A. Oxidoreductases:

1. Dehydrogenases: Nitroblue tetrazolium (NBT) method (modified after Adams, 1967; and Pearse, 1972). The following dehydrogenases were examined:

   β-hydroxybutyrate dehydrogenase

   Lactate dehydrogenase

   Malate dehydrogenase

   Succinate dehydrogenase

   Iso-citrate dehydrogenase

   Glucose-6-phosphate dehydrogenase

   Non-specific dehydrogenase (using the heart's own endogenous substrate).

2. Diaphorases or Tetrazolium reductases: Nitroblue tetrazolium (NBT) method.

- NADH (reduced nicotinamide adenine dinucleotide) diaphorase or NADH tetrazolium reductase.
- NADPH (reduced nicotinamide adenine dinucleotide phosphate) diaphorase or NADPH tetrazolium reductase.

3. Oxidases:

- Cytochrome oxidase: Naphthol-amine method (Burstone, 1959); G-Nadi reaction (after Grüff, 1923).
- Monoamine oxidase : Tetrazolium method (Glenner, Burtner and Brown, 1957).

4. Peroxidase:

Myoglobin peroxidase: Benzidine method (Drews and Engel, 1961).

B. Glycosyltransferase

Phosphorylase: Iodine method (Takeuchi and Kuriaki, 1955; Eränkö and Palkama, 1961; Ibrahim and Castellani, 1968).

C. Transferases

- Glutamic-oxaloacetic transaminase or Aspartate transaminase: Lead-oxaloacetate method (Lee, 1968).
- Creatine phosphokinase: Tetrazolium method (Sjövall, 1967).

D. Peptidase

Leucine aminopeptidase : LNA method (Nacklas, Crawford and Seligman, 1957).

E. Hydrolases

- Ali-(non-specific) esterase: α-naphthyl acetate method using hexazotized pararosanilin as coupler (Gomori, 1950; Davis and Ornstein, 1959).
- Acid phosphatase: α-naphthyl phosphate method (Burstone, 1958; modified by Barka and Anderson, 1962).

Non-enzyme histochemical methods

1 - Phloxine-tartrazine stain (Lendrum, 1947).

2 - Acid Fuchsin stain (Poley, Fobes and Hall, 1964).

3 - Haematoxylin-basic fuchsin-picric acid (HBFP) stain (Lie, Holley, Kampa and Titus, 1971).

Fluorescence method

NADH (reduced nicotinamide adenine dinucleotide) fluorescence-screening of the heart (Barlow and Chance, 1976).

Dehydrogenases

Dehydrogenases play a role in carbohydrate metabolism in which the process of oxidation of glucose can be considered as occurring in two phases, anaerobic and aerobic. The anaerobic phase involves the breakdown of glucose to glucose phosphate, which in turn is broken down in a series of reactions to pyruvic acid. This end product - pyruvic acid - is then oxidised in the aerobic phase to CO_2 and H_2O. This involves a series of reactions known as the tricarboxylic acid or Krebs' cycle.

The maximum yield of the energy derived from complete oxidation of glucose to CO_2 and H_2O is obtained by converting glucose to pyruvic acid in the cell cytoplasm, and then oxidising the pyruvic acid via acetyl CoA to CO_2 in the mitochondria by means of the tricarboxylic acid cycle and associated oxidative phosphorylation (Figure 1). Complete oxidation of one mole of glucose yields a total of 38 moles of energy-rich adenosine triphosphate (ATP). However, all cells can obtain a limited amount of energy from glucose without using molecular oxygen. This process of anaerobic metabolism or glycolysis (the Embden-Meyerhof pathway) is accomplished by a series of reactions in which a net synthesis of two molecules of ATP occur as each glucose molecule is converted to lactic acid.

The metabolism of the human heart is almost exclusively aerobic, and thus the myocardium is dependent on a continuous high rate of oxygen delivery. Aerobic metabolism is an efficient and, thereby, the chief pathway for the high level of energy production required by

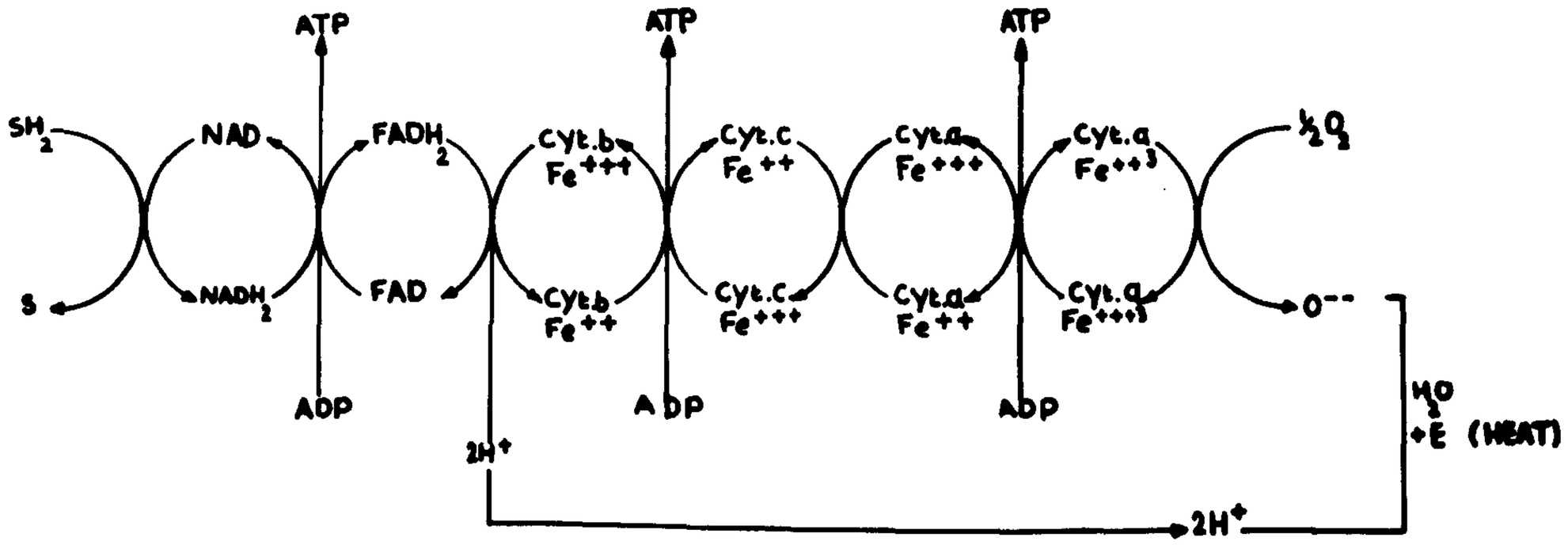


Figure 1: Oxidative phosphorylation.

The substrate SH_2 is oxidised by NAD which transfers the hydrogen atoms or electrons to FAD. The electrons are carried through the different cytochromes (cyt.) towards oxygen. Part of the energy is converted as ATP, the rest appears as heat.

the myocardium (Figure 2). Anaerobic metabolism, that is glycolysis, can be utilized only to a limited extent by the myocardium for generation of high-energy phosphate and is, therefore, insufficient in itself to maintain cardiac function (Opie, 1968; Amsterdam, 1973).

The observations that cardiac muscle relies mainly on respiration by complete oxidation of glucose through the Krebs' cycle is supported by a number of investigators who have used histochemical methods to show that heart muscle contains abundant succinate dehydrogenase (Bourne, 1953; Kent and Diseker, 1955; Wachstein and Meisel, 1955: Kobernick, Mandell, Zirkin and Hashimoto, 1963; Lushnikov, 1963; Shnitka and Nachlas, 1963; Bajusz and Jasmin, 1964a; Pearse, 1964). NADH and NADPH tetrazolium reductases, malate, glutamate, iso-citrate, lactate, B-hydroxybutyrate and alcohol dehydrogenases are also active in cardiac muscle as well as glucose-6-phosphate and 6-phosphogluconate dehydrogenases (Adams, 1967).

Principle of dehydrogenase histochemistry

Dehydrogenases are enzymes that transfer hydrogen from their specific substrates to a hydrogen acceptor. The principle of the histochemical reaction depends on the reduction of a water-soluble tetrazolium salt (redox indicator or hydrogen acceptor) to a water-insoluble formazan by the hydrogen atoms or electrons liberated during the reaction. After dehydrogenation of the substrate, electrons are transferred to the coenzyme nicotinamide adenine dinucleotide (NAD)

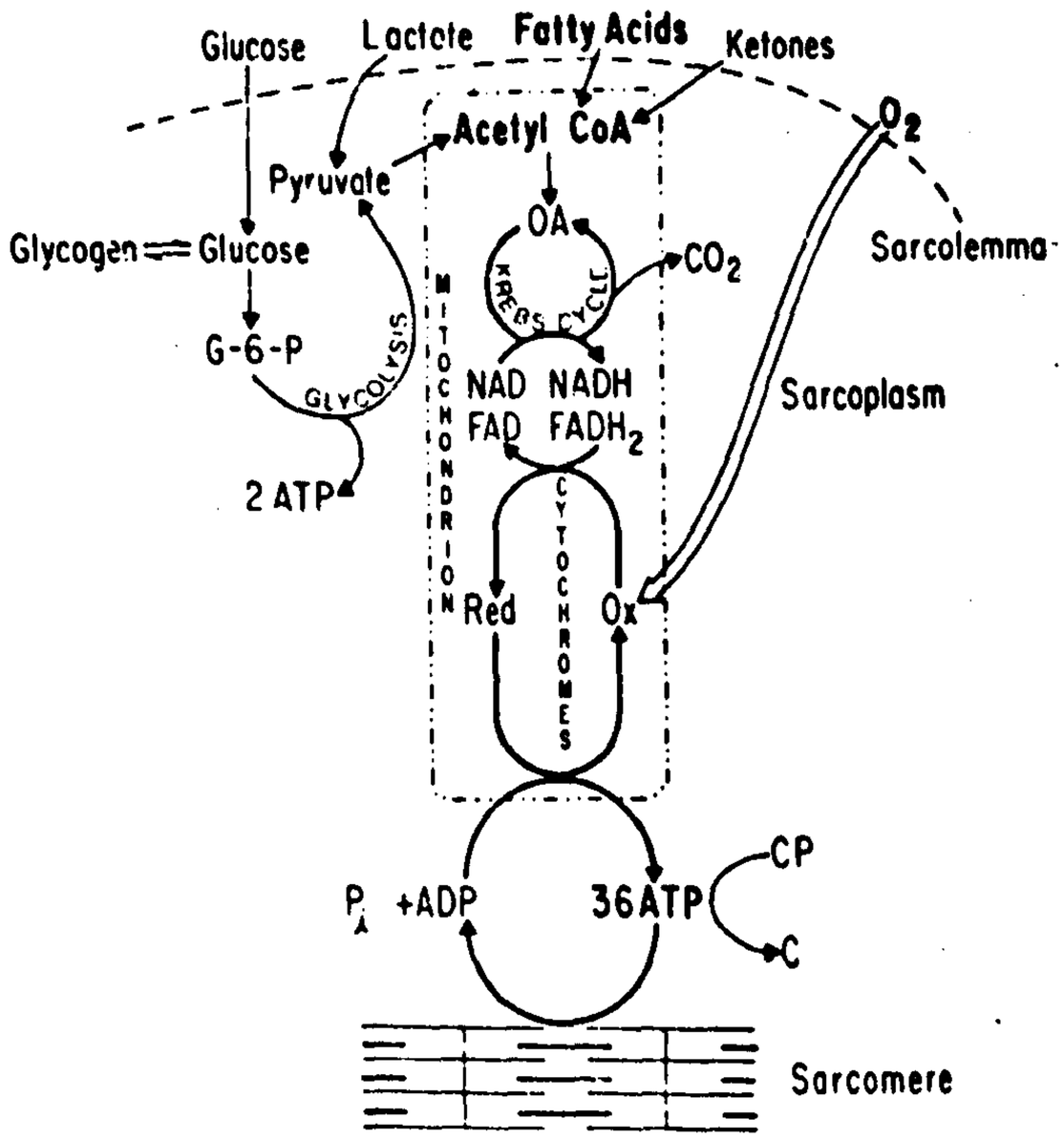


Figure 2. Diagram of metabolic pathway of energy production within the cardiac cell. Complete oxidation of a substrate such as glucose yields a total of 38 moles of ATP per mole of glucose, whereas glycolysis alone provides 2 moles of ATP.

ADP = adenosine diphosphate; ATP = adenosine triphosphate; C = creatine; CP = creatine phosphate; CoA = coenzyme A; FAD & FADH = flavin adenine dinucleotide and its reduced form respectively. G-6-P = glucose-6-phosphate; NAD & NADH = nicotinamide adenine dinucleotide and its reduced form respectively; OA = oxaloacetatic acid; OX = oxidation; PI = inorganic phosphate; Red = reduction (after Amsterdam, 1973).

or nicotinamide adenine dinucleotide phosphate (NADP) and, thence, to flavoproteins. In some instances the dehydrogenase itself can act as a hydrogen acceptor. Succinate dehydrogenase is one such enzyme, which in itself is capable of accepting hydrogen from the substrate and is then reduced by flavoproteins in a subsequent reaction. Tetrazolium salt accepts electrons between the flavoprotein and cytochrome stages of the electron transport chain (Figure 3); possibly coenzyme Q (ubiquinone) mediates transfer between flavoproteins and the tetrazole (Glenner, 1965). Cyanide is usually added to the incubating medium to block the cytochrome system, and thus encourage deviation of electrons to the tetrazolium (Rosa and Velardo, 1954).

Tetrazolium salts

In the development of histochemistry, many types of tetrazolium salts have been used for a number of techniques. They are most commonly used in the field of dehydrogenase and oxidase histochemistry. They are quaternary ammonium salts which are water soluble, nearly colourless and toxic. Under proper circumstances they are reduced to pigmented insoluble formazans.

Triphenyl tetrazolium chloride (TTC) was the first tetrazolium salt to be prepared (Pechmann and Runge, 1894). A number of workers used TTC and related compounds in the early histochemical study of animal tissues (Antopol, Glaubach and Goldman, 1948; Straus, Cheronis and Straus, 1948; Black and Kleiner, 1949; Seligman, Gofstein and

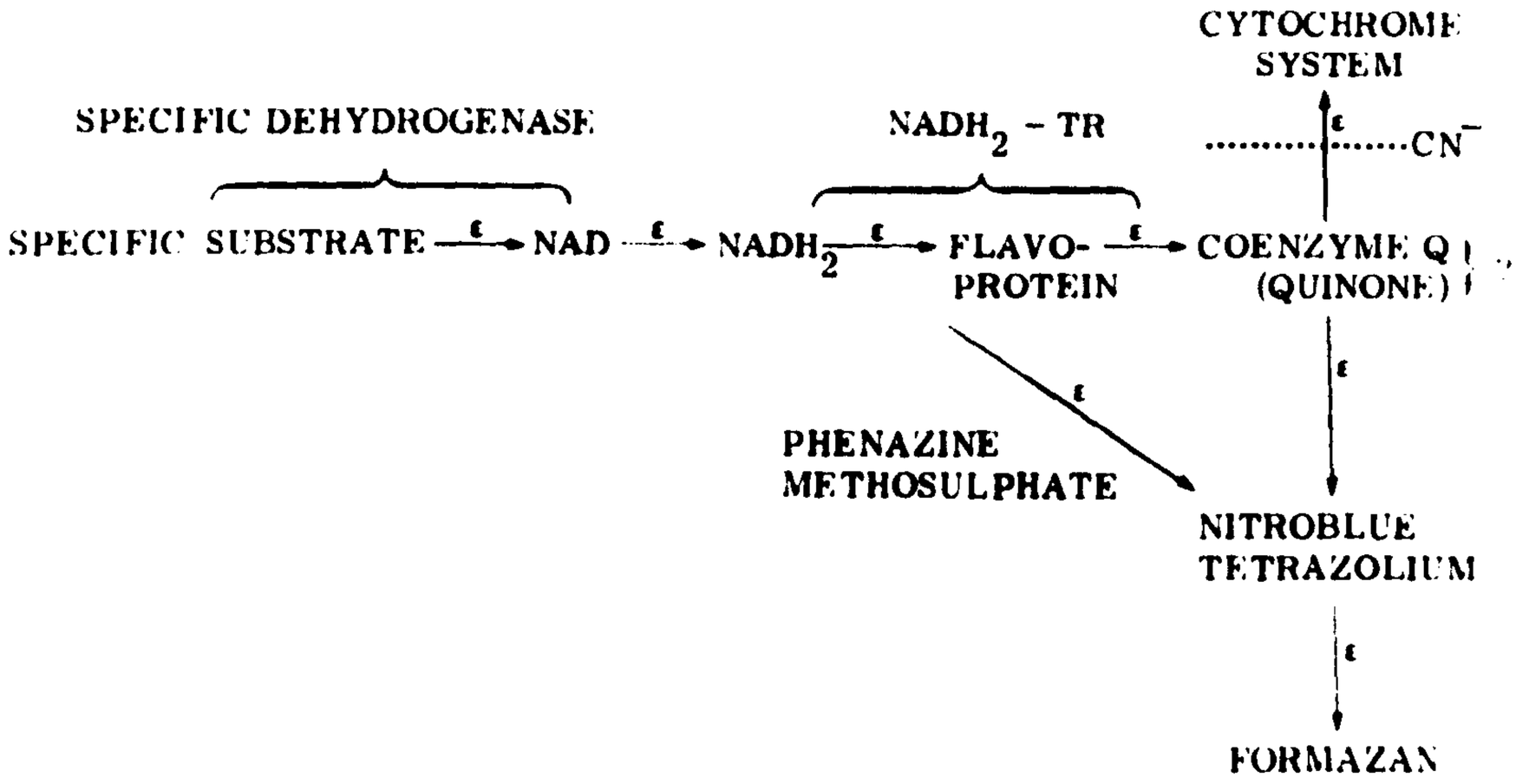


Figure 3: Mitochondrial electron transfer chain.

(⟶ = electron transfer;

after Adams, 1967).

Rutenburg, 1949; Black, Opler and Speer, 1950). Histochemically, the difficulty of obtaining a positive reaction with TTC, and the weak red colour and diffusibility of its formazan in the tissues were serious objections to its continued use (Pearse, 1972). However, at the macroscopic level, TTC was used by Sandritter and Jestädt (1957/1958), Jestädt and Sandritter (1959), Knight (1967), and Lie, Pairolero, Holley and Titus (1975) for the gross staining of the heart.

Other tetrazolium compounds were synthesized and used between the years 1950 and 1958. Examples are: neotetrazolium (NT), which was used by Antopol, Glaubach and Goldman (1948); its methoxy derivative known as blue tetrazolium (BT), which was used by Rutenburg, Gofstein and Seligman (1950); and the monotetrazolium salt INT synthesized by Atkinson, Melvin and Fox (1950).

Three other tetrazolium salts have been used in dehydrogenase histochemistry since 1957. These are the ditetrazolium chloride, nitro-BT (NBT) (Tsou, Cheng, Nachlas and Seligman, 1956; Nachlas, Tsou, de Souza, Cheng and Seligman, 1957), its tetra-nitro derivative known as tetra-nitro BT (TNBT) (Tsou, Cheng, Nachlas and Seligman, 1956; Rosa and Tsou, 1963) and the monotetrazole MTT, which was introduced by Pearse (1957). Using MTT, Scarpelli, Hess, and Pearse (1958), Hess, Scarpelli and Pearse (1958) described methods for a number of dehydrogenase enzymes.

A number of other tetrazolium compounds have been synthesized since then. However, the choice of tetrazolium salt in modern

dehydrogenase histochemistry lies between the ditetrazole nitro-BT (NBT), and the monotetrazole MTT. Desirable characteristics of tetrazolium salts for use in staining include the following (Glenner, 1965; Adams, 1967; Pearse, 1972) :-

1 - Solubility: The tetrazolium salt should be soluble enough to provide optimal concentration as an indicator.

2 - Redox potential: this represents the ease with which the tetrazolium salt is reduced. This is essential as it determines whether or not a tetrazolium salt can act as a hydrogen acceptor in the dehydrogenase system.

3 - A molecular size that permits penetration through cell membranes.

4 - No inhibition of enzyme activity.

5 - Tissue substantivity but not for special cell constituents.

6 - Lack of lipid solubility.

7 - When reduced, the developing formazan should be insoluble, forming a strongly pigmented precipitate at the site of dehydrogenation.

Nitroblue tetrazolium forms a finely granular dark blue diformazan that is "substantive" to protein. High substantivity favours improved localisation. MTT is a non-substantive tetrazole (Pearse, 1972). The diformazan of NBT, unlike the monoformazan of MTT, does not diffuse into lipids and, hence, does not give rise to artefactual localization. However, the diformazan of NBT has a tendency to precipitate at the surface of globular (hydrophobic) lipid droplets

(Novikoff, Shin and Drucker, 1961b). For this reason, some workers prefer to extract such globular lipid with a preliminary rinse in acetone (Hitzeman, 1963; Glenner, 1965; Saudek, Adams and Bayliss, 1966). It should be noted that a pink or red monoformazan can be formed from NBT which readily diffuses into lipid. This can be extracted with a final acetone rinse (Adams, 1967). The presence of the red monoformazan, which is the reduced form of the contaminating monotetrazole of NBT, is usually regarded as evidence of only weak dehydrogenase activity (Adams, Bayliss and Orton, 1967). It appears that nitroblue tetrazolium is the most satisfactory tetrazolium salt and is preferred by most workers. It is a sensitive redox indicator.

Nitroblue tetrazolium method (NBT) for dehydrogenase macroreaction

Nachlas and Shnitka (1963) applied a nitroblue tetrazolium method for the gross identification of early myocardial infarction, recommending the use of succinate as a substrate. Adams (1967) modified the method, also using succinate, as a substrate, but including the respiratory chain inhibitor, cyanide, into the incubating medium. In this present study, the nitroblue tetrazolium method is applied using various exogenous substrates and the heart's own endogenous substrate to test the histochemical activities of various dehydrogenases and their possible role in identifying early myocardial infarction in the gross at autopsy. Cyanide is added to the incubating medium as applied by Adams (1967). However, in the light of the results obtained, the method was further modified (Derias and Adams, 1978).

Pilot Studies

1. Various concentrations of nitroblue tetrazolium salt [2,2'-Di-p-nitrophenyl-5,5'-diphenyl-3,3'(3,3'-di-methoxy-4,4'-diphenylene) ditetrazolium chloride] (Sigma) were tested to determine the least concentration of tetrazolium salt required. The recommended concentration of NBT is 4 mg/ml (Pearse, 1972). An attempt to use a lower concentration and to evaluate its effect on the development of the final colour product was made by incubating normal human heart slices in NBT incubating media containing 2 mg, 1 mg and 0.5 mg/ml of the tetrazolium. The effect was tested using exogenous substrates, succinate and β-hydroxybutyrate, and the heart's own endogenous substrate. A concentration of 0.5 mg/ml proved to be satisfactory within the incubation time of 20 - 30 minutes at 37°.

2. The NBT incubating medium was buffered at various pH levels to test for optimum enzyme activity (Table 13). Normal human heart slices were incubated at different pH levels in incubating media containing either the exogenous substrate, β-hydroxybutyrate, or endogenous substrates. The rate of staining of normal myocardium and the intensity of the dark blue formazan were used as criteria to determine the optimum pH level of the dehydrogenase macroreaction.

It appeared that the dehydrogenases were active at pH levels between 6.5 and 7.5. Negligible reaction occurred at pH 9.0, while no reaction took place at pH 5.2.

Table 13. The optimum pH level of dehydrogenase macroactivity using NBT incubation media buffered at various pH levels.

Dehydrogenases tested	Enzyme macroactivity at the underlying pH levels				
	5.2	6.5	7.1	7.5	9.0
β-hydroxybutyrate dehydrogenase	-	+	+	+	$\frac{-}{+}$
Non-specific dehydrogenase	-	+	+	+	$\frac{-}{+}$

- no demonstrable enzyme activity (no staining)

+ high enzyme activity (dark blue staining)

$\frac{-}{+}$ very weak or negligible enzyme activity (pink staining).

3. The addition of polyvinylpyrrolidone (PVP) to the medium, as suggested by Novikoff (1956), exerts a protective effect on the mitochondria and, hence, increases the accuracy of localization of dehydrogenases by providing a gel-like non-electrolyte media. However, when adjacent heart-slices were incubated in NBT dehydrogenase incubating media with and without PVP, no effect was found on the macroreaction. It should be noted that newer methods (e.g. substrate film and semipermeable membranes) have been described by Lojda, Gossrau and Schiebler (1976). However, these are applicable only at the microscopic level.

Nitroblue tetrazolium (NBT) method

The following stock solutions were prepared and stored at 4^{o} for several months.

Stock Tris buffer (0.2 M) pH 7.4

a) 0.2 M Tris (hydroxymethyl) aminomethane (2.42%) 20.7 ml

b) 0.2 M HCl 79.3 ml

Stock incubating solution

a) Nitro-BT [2,2'-Di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-di-methoxy-4,4'-diphenylene) ditetrazollium chloride] (Sigma) (0.5 mg/ml). 100 ml

b) Tris buffer (pH 7.4) 100 ml

c) 5 mM $Mgcl_2$ (0.1%) 40 ml

d) Distilled water 120 ml

The pH of this stock solution was adjusted to 7.1 with 0.2 M Tris without HCl.

Stock respiratory chain inhibitor

Sodium cyanide 100 mM (0.5%) 100 ml

Stock substrate solutions

a) DL-B-Hydroxbutyric acid (Sigma),
Sodium salt (0.127 g/ml) 10 ml

b) DL-Lactic acid (Sigma)
Sodium salt (0.125 ml/ml) 10 ml

c) Sodium succinate (0.675 g/ml) 10 ml

d) L-malic acid (Sigma) (0.134 g/ml) 10 ml

e) DL-isocitric acid (Sigma) 10 ml
Trisodium salt (0.276 g/ml) 10 ml

f) D-glucose-6-phosphate (Sigma)
Disodium salt (0.304 g/ml) 10 ml

The above prepared stock solutions were stored at 4^{o} for months. Pearse (1972) recommended their storage at -20^{o}, where they can be stable for several months. In practice, it was more convenient to store them at 4^{o} for immediate use when required without the need

to wait for thawing; they were found to remain stable at 4° for three months or more. However, if any of the solutions is contaminated, it should be discarded, then freshly prepared and stored at 4°.

Incubating Media (Table 14).

Table 14. NBT incubating media for dehydrogenase macroreaction

Incubating medium	Enzymes to be demonstrated						
	B-hydroxybutyrate dehydrogenase	Lactate dehydrogenase	Succinate dehydrogenase	Malate dehydrogenase*	Iso-citrate dehydrogenase	Glucose-6-phosphate dehydrogenase	Non-specific dehydrogenase
Volume of stock solution	12 parts	12 parts	12 parts	12 parts	12 parts	12 parts	12 parts
Volume of stock substrate solution	5 parts	5 parts	5 parts	5 parts	5 parts	5 parts	-
Volume of respiratory chain inhibitor solution	5 parts	5 parts	5 parts	5 parts	5 parts	5 parts	5 parts
Volume of distilled water	-	-	-	-	-	-	5 parts
Coenzymes 100mg/100ml	NAD	NAD	-	NAD	NAD or NADP	NADP	NAD or NADP

*Malate dehydrogenase: the pH of its incubating medium when prepared becomes highly acidic. Therefore the pH should be adjusted to 7.1 before incubating the heart slices. The other enzymes do not require adjustment of the pH of their specific incubating media.

Method

Transversely sectioned heart slices, of approximately one cm thickness were rinsed in cold running water to remove blood, and were then incubated in large beakers with the various incubating media. A sufficient volume of incubating medium was used to cover completely the heart slice to a depth of 1-2 cm. They were gently agitated by a shaker to prevent stagnation of the reagents and staining artefacts.

Incubation was carried out at 37^{o} for 20 - 30 minutes. The exact time depends on the rapidity of appearance of the formazan pigment on the surface of the heart slice. The end point of incubation was when the normal part of the heart slice stained dark blue. At this point, the incubating medium was poured off and the beaker was filled with 10% formol saline. This immediately inhibited the enzyme reaction and increased the contrast between the normal and infarcted muscle. It also fixed the slice for storage.

With each heart,slices were incubated in media using the heart's own endogenous substrate and one or more of the exogenous substrates. The acetone rinse, used in histochemical practice to remove the red monoformazan (Adams, 1967) was omitted in this gross test.

The most satisfactory result was obtained when the transversely cut heart slices were incubated in a nitroblue tetrazolium (NBT) incubating medium containing both nicotinamide adenine dinucleotide (coenzyme I; NAD) and the respiratory chain inhibitor, sodium cyanide, with no added (exogenous) substrate, irrespective of the death-necropsy interval. Evidence is provided later for the role of coenzyme NAD and of cyanide on the macroscopic staining of the heart.

Nitro-BT method for dehydrogenase macroreaction

(Nachlas and Shnitka, 1963).

Nachlas and Shnitka (1963) were the first to use nitroblue tetrazolium salt, as a hydrogen acceptor, in the dehydrogenase macro-reaction for gross identification of early myocardial infarction. In the present study, the nitroblue tetrazolium method, described by these authors, was tested and compared with the current NBT method applied in this study.

Stock incubating solution

a) 1 M Sorensen's phosphate buffer (pH 7.4).

b) Nitro-BT [2, 2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride] (Sigma) (5 mg/ml).

Incubating medium

a)	Phosphate buffer at 1 M concentration	1 part
b)	Nitro-BT at 5 mg/ml	1 part
c)	water	8 parts

Method

Transversely cut heart slices are rinsed in cold water to remove traces of blood, and are then placed in the buffered tetrazolium solution for 30 minutes incubation at 37°. Normal myocardium should be stained dark blue within 15 minutes.

The same authors (Nachlas and Shnitka, 1963) recommended the addition of 0.1 M succinate to the standard buffered nitro-BT solution, when dealing with human hearts obtained after a postmortem interval of more than six hours, in order to compensate for loss of endogenous substrate from normal heart muscle.

Diaphorases (Tetrazolium Reductases)

The diaphorases, nicotinamide adenine dinucleotide diaphorase (NADH diaphorase or NADH tetrazolium reductase), and nicotinamide adenine dinucleotide phosphate diaphorase (NADPH diaphorase or NADPH tetrazolium reductase) catalyse the oxidation of the reduced coenzyme I (NADH) and coenzyme II (NADPH), respectively. As they are part of the electron transfer system, these enzymes were tested for use in the gross identification of myocardial infarction, employing NADH and NADPH as substrates - the diaphorase reaction (Adams, 1967; Pearse, 1972).

Histochemical demonstration of diaphorases

Nitroblue tetrazolium (NBT) Method.

Preparation of stock solutions

Stock 0.2 M Tris buffer (pH 7.4)

a)	0.2 M Tris (hydroxymethyl) aminomethane	20.7 ml
b)	0.2 M HCl	79.3 ml

Stock incubating solution

a)	Nitro-BT [2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-di-methoxy-4,4'-diphenylene) detetrazolium chloride] (Sigma) (0.5 mg/ml)	100 ml
b)	Tris buffer (pH 7.4)	100 ml
c)	5 m M $MgCl_2$ (0.1%)	40 ml
d)	Distilled water	120 ml

The pH of this stock solution is adjusted to pH 7.1 with 0.2 M Tris without HCl.

Stock respiratory chain inhibitor

Sodium cyanide 100 m M (0.5%)	100 ml

These stock solutions are stored at 4^{o}.

Substrates

a) β-nicotinamide adenine dinucleotide (B-NAD: Sigma)

b) Nicotinamide adenine dinucleotide phosphate (NADP; Sigma).

Incubating Medium (Table 15).

Table 15. Nitroblue tetrazolium (NBT) incubating medium for diaphorases

Enzyme	Volume of stock NBT solution	Volume of distilled water	Volume of sodium cyanide	Amount of substrate
NADH diaphorase	12 parts	5 parts	5 parts	200 mg per 100 ml
NADPH diaphorase	12 parts	5 parts	5 parts	200 mg per 100 ml

Method

- Transversely cut heart slices (approximately 1 cm thick) were briefly rinsed in cold running water to remove traces of blood from the cut surface.

The slices were incubated in the NBT incubating medium for diaphorases,using sufficient volume completely to cover the slice to a depth of 1 - 2 cm; they were then gently agitated by a shaker to prevent staining artefacts.

Incubation was carried out at 37^{o} for 20 - 30 minutes. The exact time depended on the first appearance of the dark blue formazan pigment on the surface of the slice. The incubating medium was then poured off and replaced by 10% formol saline, which immediately inhibited the enzyme reaction and increased the contrast between the normal and necrotic muscle. It also fixed the heart slice for storage.

The acetone rinse, used to remove the red monoformazan in microscopic histochemistry (Adams, 1967), was not applied in this macroscopic staining.

Intermediate electron acceptor-phenazine methosulphase (PMS)

The rate of transfer of electrons to tetrazolium salt by the action of specific dehydrogenases can be limited by the activity of the intermediate enzymes, NADH or NADPH tetrazolium reductases, which are an integral part of the electron transfer chain. Farber and Bueding (1956) recommended the use of phenazine methosulphate (PMS) as an intermediate electron acceptor to accept electrons directly from the reduced coenzymes (or flavoproteins) and transfer them directly to the tetrazolium salt (Figure 3) without need for mediation of the nucleotide tetrazolium reductases, thus greatly accelerating the tetrazolium reaction of the dehydrogenases. Brody and Engel (1964) emphasise the addition of cyanide to the incubating medium when PMS is used, because the latter agent probably accelerates electron transfer to the cytochrome system as well as to the tetrazole.

In the present study, the role of PMS in accelerating the dehydrogenase macroreaction was tested by incubating heart slices in the NBT incubating media described for the dehydrogeanses, with and without added substrates, to which PMS was added at a concentration of 1 mg/ml of medium. Incubation was carried in the dark because PMS is sensitive to light.

Dehydrogenase inhibitors

The inhibition of the formation of the formazan pigment in the NBT dehydrogenase macroreaction was tested with the following inhibitors added to the incubating media:

p-chloromercuribenzoate	(0.001 M)
N-ethyl maleimide	(0.01 M)
malonate	(0.01 M)
oxaloacetate	(0.01 M)
oxalate	(0.01 M)
iodoacetate	(0.01 M)

Cytochrome Oxidase

Cytochrome oxidase is concerned in the main oxidation pathway. In parallel with the high activity of dehydrogenase in cardiac muscle, cytochrome oxidase activity is also conspicuous in human heart muscle, being closely attached to cell mitochondria (Burstone and Miller, 1961). Microscopically, Morales and Fine (1966) observed an early depletion of cytochrome oxidase in recent human myocardial infarction. Macroscopically, the histochemical demonstration of cytochrome oxidase is attempted in this study, to investigate the possible use of the enzyme macroreaction in identifying early myocardial damage in the gross.

Two histochemical methods for macroscopic demonstration of cytochrome oxidase in human heart slices were applied. The naphtholamine method (Burstone, 1959), and the G-Nadl oxidase reaction (after Gräff, 1923). The latter method stained normal heart muscle dark blue and is the one described below. The principle of the reaction is based on the fact that cytochrome oxidase will act as a catalyst for the oxidation reaction between ∝-naphthol and dimethyl-p-phenylamine-diamine hydrochloride to form indophenol blue (Nadl reaction).

G-Nadl reaction (after Gräff, 1923; Culling, 1974).

∝-naphthol solution

0.1 g of ∝-naphthol is dissolved in 1 ml of alcohol, and then made up to 100 ml with distilled water.

Oxidase Reagent

0.12 g of dimethyl-p-phenylaminediamine hydrochloride was dissolved in 100 ml of distilled water. This solution should be colourless, or just tinted, otherwise it should be discarded.

Nadi Reagent

20 ml each of ∝ -naphthol solution and oxidase reagent were mixed, and 8 ml of 0.1 M phosphate buffer pH 7.4 was added to the mixture.

Control

Potassium cyanide (3.25 mg/50 ml) was added to control Nadi reagent.

Method

Transversely cut heart slices were incubated in Nadi reagent at 37°. Dark blue staining of the heart slice occurred in 2 - 5 minutes.

Monoamine Oxidase

Monoamine oxidase (MAO) activity has been identified histochemically by Pearse (1964) in the sarcoplasm, particularly in the mitochondria, of myocardial fibres in biopsies of human right ventricle. Differences in the distribution of the enzyme in the hearts of different species were recorded by Müller and Pearse (1965). An early loss of MAO activity in experimental myocardial infarction in rats has been reported by Bajusz and Jasmin (1964a). However, the activity of this enzyme was not investigated in human myocardial infarction, either microscopically or macroscopically. In the present study, the histochemical demonstration of MAO activity was tested in human hearts, at the macroscopic level, to see whether it was of use as an indicator of early myocardial damage.

The method applied was that of Glenner, Burtner and Brown (1957). The technique depends on enzymatic oxidation of the tryptamine substrate to aldehyde. The formed aldehyde then, non-enzymatically, reduces the tetrazolium salt to insoluble pigmented formazan.

Tetrazolium method (Glenner, Burtner and Brown (1957)

Incubating solution:

Tryptamine hydrochloride [3-(2-Aminoethyl) indole HCl] (Sigma)	25 mg
Sodium sulphate	4 mg
Nitroblue tetrazolium [2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'dimethoxy-4,4'diphenylene) ditetrazolium chloride] (Sigma)	5 mg

0.1 M phosphate buffer pH 7.6	5 ml
Distilled water	15 ml

Method

Transversely cut heart slices were briefly rinsed in cold running water to remove traces of blood from the cut surface, and were incubated in the above incubating solution at 37^{o} for 45 minutes. They were gently agitated to prevent stagnation of the reagent and staining artefacts. Although the heart slices should be stained blue, they reacted reddish-brown.

Myoglobin Peroxidase

A recent contribution to the clinical diagnosis of myocardial infarction is the reported rise in the serum level of the respiratory pigment, myoglobin, in patients with acute myocardial infarction (Stone, Waterman, Harimoto, Murray, Wilson, Platt, Blomoqvist, and Willerson, 1977). Accordingly, an attempt was made here at the histochemical demonstration of myoglobin in transverse heart slices.

The method employed was the benzidine method (Drews and Engel,1961). The principle of the reaction is that peroxidase activity of myoglobin in muscle catalyses the transfer of oxygen from hydrogen peroxide to benzidine with a resulting blue precipitate at the site of enzyme activity. The method is now outdated because of the legal restriction in handling carcinogens.

Benzidine method (Drews and Engel, 1961)

Preparation of benzidine solution (modified Van Duijn solution):

a) 500 mg benzidine are dissolved in 100 ml of 0.85% NaCl at 80°. Cool to room temperature and filter.

b) To 36 ml of benzidine solution, add 4 ml saturated (about 40%) ammonium chloride solution and 3 drops (about 0.15 ml) 3% hydrogen peroxide solution (freshly diluted from the stock 30% solution). This benzidine solution should be made fresh every time).

Method

a - Transversely cut heart slices were blotted dry to remove traces of blood. Rinsing in water was avoided as myoglobin is water soluble

(Drews and Engel, 1961; James, 1968). Instead, the slices were fixed in 10% formol saline for 30 minutes.

b - The heart slices were placed in benzene (sic) for one minute.

c - They were stained 5 - 8 minutes in the modified Van Duijn solution given above, and were then rinsed in 0.85% saline solution.

Result

Dark blue deposits indicate the site of enzymatic activity. Macroscopically, only a few isolated myocardial fibres were stained light blue. No proper staining of the myocardium was seen. The expected complication of a reaction with haemoglobin peroxidase was not apparent.

Phosphorylase

The activity of this enzyme has been studied in human myocardial infarction by Morales and Fine (1966), but only at the microscopic level. Because of its major role in controlling glycogen metabolism, it was tried in the present work as a test in the gross for early myocardial infarction.

The technique used was the iodine method (Takeuchi and Kuriaki, 1955; Eränkö and Palkama, 1961; Ibrahim and Castellani, 1968). The substrate contains glycogen and glucose-1-phosphate. The phosporylase (if present) enlarges the glycogen molecule by accretion of glucose units from the glucose-1-phosphate. The extended and branched molecules of glycogen are demonstrated by their reaction with iodine, which stains the linear form blue (action of α-glucan phosphorylase) and the branched form red brown (action of α -glucan branching glycosyltransferase). Takeuchi and Kuriaki (1955) introduced the use of activators, insulin and adenosine phosphate, and glycogen as a primer. Ibrahim and Castellani (1968) employed various nucleotides as activators, and found that the most intense reaction was obtained when the medium contained AMP (adenosine monophosphate) as activator. Eränkö and Palkama (1961) recommended the addition of PVP (polyvinylpyrollidone) as a stabilizing agent because phosphorylase is a soluble enzyme and because the low molecular weight unbranched glycogen product is diffusible.

Iodine method (Takeuchi and Kuriaki, 1955; Eränkö and Palkama, 1961; Ibrahim and Castellani, 1968).

Preparation of stock solution

0.1 M acetate buffer (pH 5.9)	100 ml
Glucose-1-phosphate	1 g
Adenosine-5-phosphate	100 mg
Glycogen	20 mg
Sodium fluoride	1.8 g
Polyvinylpyrrolidone	9 g
Insulin (40 i.u./ml)	10 drops

The reagents were added to 100 ml 0.1 M acetate buffer (pH 5.9) in the above order. It was not filtered. This solution keeps several months at 0 - 4°.

Incubating medium

A - For phosphorylase enzyme: 10 ml absolute ethanol were added to 50 ml of freshly filtered stock solution.

B - For phosphorylase + branching enzyme use filtered stock solution only.

Methods

a) Heart slices were incubated for 1-2 hours at 37° (phosphorylase), and for 30 minutes at 37° (phosphorylase + branching enzyme); they were gently agitated to prevent stagnation of the reagent.

b) They were briefly washed in 40% ethanol, fixed for one hour in absolute ethanol, and were then stained in dilute Gram's iodine (1:10) for 5 minutes.

Result: At the macroscopic level, enzyme activity could not be demonstrated.

Glutamic-Oxaloacetic Transaminase

(Aspartate Transaminase)

Acute myocardial infarction is associated with a rise in the serum level of glutamic-oxaloacetic transaminase, GOT (or aspartate transaminase, AST) (LaDue, Wróblewski and Karmen, 1954). The histochemical demonstration of the activity of this enzyme, which is most concentrated in cardiac muscle (Cohen and Hekhuis, 1941), has not been investigated, so far, in human or experimental myocardial infarction. In the present study, an attempt is made to macroscopically demonstrate the activity of this enzyme in heart muscle, and to investigate its possible role in identifying acute myocardial infarction at necropsy, as it does clinically.

Lee (1968) introduced a histochemical method to demonstrate GOT activity in fresh cryostat sections of rat heart. The method was then modified by Lee and Torak (1968 a, b). The method is based on the enzymatic precipitation of lead oxaloacetate, and the subsequent formation of visible black lead sulphide at site of enzymatic activity.

Lead-oxaloacetate method (Lee, 1968)

Incubating solution

a) 266.2 mg of L-aspartic acid and 58.4 mg of α-ketoglutaric acid were dissolved in 10 ml of distilled water and 25 ml of 0.2 M imidazole.

b) The pH was adjusted to 7.5 with 0.2 N NaOH.

c) Distilled water was added to bring the volume to 50 ml.

d) Shortly before use, an equal amount of 12 mM lead nitrate solution was added, dropwise, with constant stirring.

e) The final pH should be 7.2 to 7.4

Method

a) Transversely cut heart slices were fixed in cold acetone (4^{o}) for 4 hours.

b) They were washed briefly in distilled water, and were incubated at room temperature for one hour in the above incubating solution. They were gently agitated to prevent stagnation of the reagent.

c) The medium was filtered when cloudiness resulted.

d) The heart slices were briefly washed in four changes of distilled water, and were immersed in 1% ammonium sulphide for 1 to 2 minutes.

e) They were washed in cold running water for 30 minutes, fixed and stored in 10% formol saline.

Result: The presence of GOT activity is indicated by a dark brown to black precipitate of lead sulphide. After the addition of the 1% ammonium sulphide (step (d)), the endocardium immediately stained black. The myocardium also stained black in the gross, but the stain was non-homogenous and patchy, making it difficult to assess the result precisely.

Creatine Phosphokinase

In the laboratory diagnosis of acute myocardial infarction, creatine phosphokinase (CPK) was found to be the first to respond, with a rise in its serum level within 3 to 6 hours after infarction (Hess, MacDonald, Frederick, Jones, Neely and Gross, 1964). Its release into the circulation has been correlated with histologically demonstrable myocardial necrosis (Kjekshus, 1976); but the histochemical demonstration of CPK activity in infarcted human myocardium was not attempted. A recent study by Anderson, Popple, Parker, Sayer, Trickey and Davies (1979) revealed an early macroscopic loss of enzyme activity in experimental myocardial infarction in dogs. In the present study, the histochemical demonstration of CPK in the gross is tested in transverse human heart slices to assess its possible role in identifying early human myocardial infarction.

The histochemical method used in this study is the tetrazolium method of Sjövall (1967), originally designed for the localization of the enzyme in rat and human skeletal muscle using fresh cryostat sections. The histochemical localization of CPK activity is based on the formation of blue coloured formazan through the reduction of nitroblue tetrazolium. Sjövall (1967) described the following reactions in the demonstration of CPK:

(1) Phosphocreatine + ADP $\xrightarrow{\text{creatine kinase}}$ creatine + ATP

(2) ATP + glucose $\xrightarrow{\text{Hexokinase}}$ Glucose-6-phosphate + ADP

(3) Glucose-6-phosphate + NADP $\xrightarrow{\text{Glucose-6-phosphate dehydrogenase}}$ $NADPH_2$ + 6-phosphogluconic acid

(4) $NADPH_2$ + PMS ⟶ NADP + reduced PMS

(5) Reduced PMS + NBT ⟶ reduced NBT (formazan) + PMS

Reactions (2, 3) were proposed by Kornberg (1950) for ATP (adenosine triphosphate) determination, and the combination of these with (1) was suggested by Oliver (1955) for the spectrophotometric measurement of CPK activity.

From the equations described above, it will be observed that in equation (1), the enzyme creatine kinase in the tissue catalyses the formation of ATP from phosphocreatine used as a substrate. In equation (2), glucose is converted to glucose-6-phosphate by the activity of hexokinase (endogenous and exogenous). Reaction (3) involves the oxidation of glucose-6-phosphate to 6-phosphogluconic acid. During this process, glucose-6-phosphate dehydrogenase (endogenous and exogenous) also transfers electrons to NADP (nicotinamide adenine dinucleotide phosphate) included in the incubating medium which becomes reduced to $NADPH_2$. In equation (4), PMS (phenazine methosulphate) in the incubating medium acts as an intermediate electron acceptor, transferring the electrons from $NADPH_2$ to nitroblue tetrazolium (NBT). The latter is then reduced to the blue formazan pigment shown in reaction (5).

If PMS is omitted in reaction (4), the electrons will then be transmitted from $NADPH_2$, by the activity of the tissue $NADPH_2$-tetrazolium reductase, to nitroblue tetrazolium. The latter is then reduced to dark blue formazan.

Tetrazolium method (Sjövall, 1967)

Transversely cut heart slices are rinsed in cold running water to remove traces of blood from the cut surface, and are incubated at room temperature for 30 minutes in the following incubating medium:

Triethanolamine-acetic buffer 0.2 M pH 6.9

Magnesium acetate 0.02 M

Glucose 0.02 M

This solution contained per 30 ml.

Phosphocreatine	36 mg
Adenosine 5'-diphosphate	12 mg
NADP (nicotinamide adenine dinucleotide phosphate)	6 mg
Hexokinase 15 ul	60 mg
Glucose-6-phosphate dehydrogenase 15 ul	30 mg
PMS (phenazine methosulphate)	3 mg
NBT (nitroblue tetrazolium) [2,2'-di-p-nitrophenyl-5,5' diphenyl-3,3'(3,3'-di-methoxy-4,4'-diphenylene) ditetrazolium chloride] (Sigma)	3 mg

It was observed from the above comment on the equations involved in this method, that the final pathway for the demonstration of CPK activity is by the way of the tetrazolium reductase. To test whether the localization of the final reaction product (FRP) is demonstrating the activity of CPK, heart slices were incubated in the same incubating medium described above, but omitting the substrate phosphocreatine. Heart slices were also incubated in the above incubating medium with and without including PMS.

Sjövall (1967) suggested that a good result is obtained when sections were exposed to ice-cold 10% formalin for 10 minutes, briefly washed in an ice-cold 70% ethanol solution, and then left for 10 minutes in another similar solution. The sections were then washed for 10 minutes in cold running tap water before they were incubated in the incubating medium. If non-fixed sections are used, sodium cyanide is added in order to block the respiratory chain.

Aminopeptidase

Normal myocardial fibres contain no histochemically demonstrable aminopeptidase activity. In normal myocardium the enzyme is confined to mast cells (Monis and Weinberg, 1964). The aminopeptidase that appears during infarction is at first located in the polymorphonuclear leucocytes which infiltrate the lesion about twelve to twenty-four hours after coronary occlusion. The histochemical demonstration of aminopeptidase activity in human myocardial infarction in the gross has not been reported in the literature. It is attempted in this present study in myocardial infarcts of clinical age twelve to twenty-four hours, to test whether the macroreaction can be diagnostic for myocardial damage at this time.

The histochemical method used is the LNA method for leucine aminopeptidase (Nachlas, Crawford and Seligman, 1957). The histochemical technique involves the simultaneous coupling of the enzymically liberated β-naphthylamine (formed by the hydrolysis of the substrate β-naphthylamide) with a suitable stable chromogenic diazonium salt, such as Fast blue B salt.

LNA method for leucine aminopeptidase

(Nachlas, Crawford and Seligman, 1957).

Stock substrate solutions

0.8% L-leucyl-B-naphthylamide

0.8% L-leucyl-4-methoxy-B-naphthylamide.

These solutions can be stored at 0 - 4° for several months.

Incubating medium

Stock substrate solution	2 ml
0.1 M acetate buffer (pH 6.5)	20 ml
0.85% sodium chloride	16 ml
Potassium cyanide (20 mM)	2 ml
Fast blue B salt	20 mg

Method

Heart slices with myocardial infarction of estimated clinical age of twelve to twenty-four hours were incubated in the incubating medium, using both substrates separately, at 37^{o} for 4 hours and were gently agitated to prevent stagnation of the reagent.

Result

The presence of aminopeptidase is indicated by a red precipitate, but no macroscopic enzyme activity could be demonstrated.

Allesterase

Moderate non-specific esterase activity is located in the peri-nuclear region of normal myocardial fibres (Pearse, 1964). Increased non-specific esterase activity was reported by Morales and Fine (1966) in their detailed investigations on the histochemical characteristics of early human myocardial infarction. Macroscopically, the histochemical demonstration of this enzyme activity has not previously been reported in human or experimental myocardial infarction. In the present study, an attempt to demonstrate the activity of non-specific esterase in transverse heart slices is made to test the possible role of the enzyme in revealing early myocardial damage in the gross.

The method applied is the α-naphthyl acetate method for non-specific esterase using hexazotized pararosanilin (Gomori, 1950; Davis and Ornstein, 1959). Other substrates are available with substituted naphthol. The incubating medium contains α-naphthyl acetate dissolved in acetone; hexazonium pararosanilin is included in the medium as coupler. The esterase activity in the tissue splits the α-naphthyl acetate, releasing α-naphthol. This is known as the primary reaction product (PRP). This then combines with hexazonium pararosanilin to produce an insoluble azo dye at the site of enzyme activity. This last is the final reaction product (FRP).

α-naphthyl acetate method for non-specific esterase using hexazotized pararosanilin (Gomori, 1950; Davis and Ornstein, 1959).

Substrate solution

1% α-naphthyl acetate in acetone.

Buffer solution

0.2 M phosphate buffer stock solution (Na_2HPO_4).

Hexazotized Pararosanilin: HPR (Davis and Ornstein, 1959).

1- Dissolve 1 g pararosanilin hydrochloride in 20 ml of distilled water and add 5 ml concentrated HCl. Warm gently, cool and filter. Store in dark, preferably at 4^{o}.

2- 4% aqueous solution of sodium nitrite is freshly prepared.

For use mix equal parts of the two solutions, shake for a few seconds until the colour becomes amber. Adjust to pH 5.0 by the addition of a few drops of normal NaOH.

Incubating medium

To 15 ml of 0.2 M phosphate buffer stock solution add 5 ml of distilled water and 0.5 ml substrate solution. Shake and add 1.6 ml of HPR.

Method

Heart slices were incubated in the above incubating medium at room temperature for 1 to 5 minutes. The heart slices stained dark reddish brown.

The colour of the FRP was so intense that contrast of colour between increased activity in myocardial infarction and normal myocardium was not distinct, making it difficult to outline areas of myocardial damage.

Acid Phosphatase

Acid phosphatase activity was demonstrated histochemically by Pearse (1964), in the perinuclear region of normal myocardial fibres. Morales and Fine (1966), in studying the histochemical characteristics of early human myocardial infarction, observed that myocardial fibres did not show any microreaction for acid phosphatase. The histochemical demonstration of the lysosomal enzyme phosphatase was attempted in the present study in myocardial infarction to test the possible use of the enzyme macroreaction in identifying areas of myocardial damage.

The method applied is the α-naphthol phosphate method using hexazonium pararosanilin (Burstone, 1958; modified by Barka and Anderson, 1962). The principle of the method is the enzymatic hydrolysis of the substrate α-naphthyl phosphate at acid pH, and the simultaneous coupling of the liberated naphthol and the hexazotised pararosanilin. Variants of this method depend on the use of substituted naphthols (as naphthol AS-TR phosphate). However, Barka and Anderson (1962) recommended the use of α-naphthyl phosphate method with tissues that have a low enzyme concentration.

α-Naphthyl phosphate method using hexazonium pararosanilin (Burstone, 1958; modified by Barka and Anderson, 1962).

Preparation of stock solutions

a) pararosanilin solution:

Dissolve 1 g pararosanilin hydrochloride in 20 ml of distilled water and 5 ml concentrated HCl with gentle warming.

Filter after cooling and store at room temperature.

b) Sodium nitrite solution (4%): this should preferably be freshly prepared.

c) Michaelis veronal acetate buffer stock solution:
9.714 g sodium acetate + 14.714 g sodium barbiturate in distilled water to a final volume of 500 ml.

d) Substrate solution:
Dissolve 400 mg sodium α-naphthyl phosphate in 100 ml Michaelis buffer stock solution (c). Store at 4^{o}.

Incubating medium

α-Naphthyl phosphate hexazonium pararosanilin:
Mix 1.6 ml of (a) and (b) in a test tube at room temperature (the hexazonium pararosanilin solution).

Pour this mixture into 10 ml of substrate solution (d), previously diluted with about 20 ml distilled water.
Adjust to pH 6 with N NaOH. Add distilled water to a final volume of 40 ml.

Method

- Heart slices with myocardial infarction of estimated clinical age of twelve to twenty-four hours were fixed in formol sucrose at 4^{o} for 2 hours.

- They were rinsed and stained in the above incubating medium for 30 - 90 minutes at room temperature, away from direct light. They were gently agitated to prevent stagnation of the reagent.

- They were then rinsed in water and fixed in 10% formol saline.

Result

Homogenous, non-specific red staining of the heart slices occurred.

Summary on macroenzymatic techniques

Table 16 summarizes the enzymes examined, the methods applied, and the result of the macroenzymatic reaction on the gross staining of the heart. A preliminary review of these methods in relation to detecting areas of recent myocardial infarction is set out now, mainly to eliminate unsuitable methods at this point.

Of the various enzymes tested, the dehydrogenases and diaphorases, using nitroblue (NBT) method, are the most sensitive indicators in depicting early myocardial damage in the gross. The incubating medium for non-specific dehydrogenase enriched with added coenzyme I (NAD; nicotinamide adenine dinucleotide) but no added specific substrate was found to give optimal results for the gross detection of myocardial infarction. The inclusion of cyanide in the incubating medium to direct electron transfer away from the cytochrome oxidase system has increased the final colour product and increased the consistency of results.

Failure to demonstrate the macroactivity of some of the enzymes examined, and the non-specific staining of the heart with the others will be discussed in Chapter V.

Microscopic examination of infarcts

Microscopic enzyme histochemistry was not used in this work for two main reasons. Firstly, the main object of this study was the identification of early myocardial infarction in the gross, which is a problem of much concern to pathologists. Secondly, microscopic

enzyme histochemistry is essentially confirmatory and is useless if the correct area is not chosen. Preliminary studies suggested that subsequent microscopic confirmation is more reliable with conventional non-enzymic methods. Furthermore, enzyme histochemistry requires frozen tissue and early technical processing which introduces a difficulty when the rest of the methods are based on formalin-fixed tissue.

Table 16: Enzyme macroreaction investigated for the identification of early human myocardial infarction

Enzyme	Method	Macroscopic staining of the heart (colour of F.R.P.)	
		Normal myocardium	Myocardial infarction (up to clinical age of 24h)
Dehydrogenases: non-specific dehydrogenase β-hydroxybutyrate dehydrogenase Lactate dehydrogenase Malate dehydrogenase Succinate dehydrogenase Iso-citrate dehydrogenase Glucose-6-phosphate dehydrogenase	NBT method	dark blue	pink
Diaphorases: NADH diaphorase NADPH diaphorase	NBT method	dark blue	pink
Cytochrome oxidase	G-Nadi reaction	dark blue	light blue
Monoamine oxidase	Tetrazolium method	reddish brown	light brown
Myoglobin peroxidase	Benzidine method	no stain	

Table 16: Enzyme macroreaction investigated for the identification of early human myocardial infarction

Enzyme	Method	Macroscopic staining of the heart (colour of F.R.P.)	
		Normal myocardium	Myocardial infarction (up to clinical age of 24h)
Phosphorylase	Iodine method	No stain	
Glutamic-oxaloacetic transaminase	Lead oxaloacetate method	non-homogenous, patchy dark brown stain	
Creatine phosphokinase	Tetrazolium method	dark blue	pink
Aminopeptidase	LNA method	No stain	
Non-specific esterase	α-naphthyl acetate method using hexazonium pararosanilin	dark reddish brown	
Acid phosphatase	Naphthyl phosphate method using hexazonium pararosanilin	homogenous, non-specific red stain	

Non-enzyme histochemical methods

The following histochemical methods were applied to formalin-fixed, paraffin-embedded heart sections. The material was obtained from the hearts examined for macroscopic enzyme histochemistry and consisted of the mirror-image slice of the infarct revealed by the macroenzymatic reaction, and one or two blocks of the adjacent normal myocardium.

1. Haematoxylin and eosin.
2. Phloxine-tartrazine stain (Lendrum, 1947).
3. Acid Fuchsin stain (Poley, Fobes and Hall, 1964).
4. Haematoxylin-basic fuchsin-picric acid (HBFP) stain (Lie, Holley, Kampa and Titus, 1971).

Phloxine-tartrazine stain

This stain was designed by Lendrum (1947) as a general histologic stain, and also for the demonstration of inclusion bodies.

Method

1. Deparaffinize tissue sections and hydrate to distilled water.
2. Stain lightly with alum haematoxylin.
3. Blue in running tap water.
4. Stain with 0.5% phloxine in 0.5% calcium chloride for 30 minutes.
5. Rinse in water, drain almost dry.
6. Flood with a saturated solution of tartrazine in Cellosolve (ethylene glycol, monomethyl ether) which differentiates and counterstains.

7. Rinse with 95% alcohol.

8. Dehydrate, clear, and mount in D.P.X.

Result

Normal myocardium stains yellow.

Ischaemic myocardium stains bright red.

Acid fuchsin stain

The method was designed by Selye (1958) to evaluate toxic cardiac necrosis in experimental animals, and was modified by Poley, Fobes and Hall (1964) to detect early myocardial infarcts.

Method

Staining solution:

Solution A

This consists of 10 ml cresyl violet solution, 40 ml distilled water, and 0.2 ml oxalic acid (1%). The stock solution consists of 1 g in 500 ml distilled water, filter after one hour. The solution should be freshly prepared.

Solution B

This is 1% phosphotungstic acid, filtered.

Solution C

This consists of 20 ml acid fuchsin (0.01%), 15 ml orange G (0.01%), 15 ml methyl green (0.01%), and 0.2 ml oxalic acid (1%).

Staining Procedure

1. Deparaffinize tissue sections and hydrate to distilled water.
2. Stain 15 minutes in Solution A.
3. Rinse in running water 10 minutes.
4. Mordant 15 minutes in Solution B.
5. Rinse in running water for 3 minutes.

6. Stain in Solution C for approximately 30 minutes in a 60°C oven, moving the slices frequently.
7. Rinse in glacial acetic acid (0.5%).
8. Dehydrate in alcohol.
9. Clear in xylol and mount in D.P.X.

Result

Normal myocardium stains blue green.

Ischaemic myocardium stains dark red.

Haematoxylin-basic fuchsin-picric acid (HBFP) stain

This method was designed by Lie, Holley, Kampa and Titus (1971), for the histochemical demonstration of early myocardial ischaemia.

Method

Staining solution:

Solution A

Alum haematoxylin. Mix 6 gm of aluminium ammonium sulphate, 0.5 g of haematoxylin, and 0.25 g of yellow mercuric oxide in 70 ml of distilled water.

Boil for 10 minutes, cool, and then add 30 ml of glycerin and 4 ml of glacial acetic acid.

Filter before use.

Solution B

Basic fuchsin. This is a 0.1% solution in distilled water.

Solution C

Picric acid. This is a 0.1% solution in absolute acetone.

Staining Procedure

All steps are carried out at room temperature. Solution C and all rinsing fluids are discarded after each batch of five to six tissue sections.

1. Deparaffinize tissue section and hydrate to distilled water.
2. Stain in Solution A for 10 seconds.
3. Wash in running cold tap water for 5 minutes.
4. Stain in Solution B for 3 minutes.
5. Rinse briefly (5 to 10 seconds) in distilled water.
6. Rinse briefly (5 to 10 seconds) in absolute acetone.
7. Differentiate in Solution C until the red (basic fuchsin) colour ceases to run off the section - usually about 20 seconds.
8. Rinse briefly (5 to 10 seconds) in absolute acetone.
9. Clear in xylol and mount in D.P.X.

Result

Normal myocardium stains light brown.

Ischaemic myocardium stains crimson red.

NADH Fluorescence Method

NADH fluorescence-screening test of the heart

(after Barlow and Chance, 1976).

Barlow and Chance (1976) reported on fluorescence emission of reduced nicotinamide adenine dinucleotide (NADH) from the surface of perfused rat hearts, where ischaemic areas can be measured by NADH fluorescence photography. Local ischaemia resulted in a corresponding well defined region of high NADH fluorescence in the perfused rat heart.

On this principle, it was thought that ischaemic areas in human myocardium might well behave similarly, and could be outlined by fluorescence of endogenous NADH (if present).

Method

Transversely cut heart slices from suspected cases of recent myocardial infarction were examined in a dark room, using a UV lamp at 254 or 354 mU. Areas of myocardium showing fluorescence were recorded, and the heart slices were then stained with the NBT method. In only a few cases did the fluorescent areas correspond with ischaemic areas outlined by the gross enzyme histochamistry.

Although the technique is a simple one, yet considerable experience is required to pick out ischaemic areas, and more confirmatory work is needed. Also, photographic documents of specimens were difficult to obtain as fluorescence photographs require the use of a special camera.

CHAPTER IV

RESULTS

I. GENERAL CONSIDERATION

A. Differential histochemical staining of the myocardium

Of the various macroscopic histochemical methods tested, the nitroblue tetrazolium (NBT) method for dehydrogenases and diaphorases (tetrazolium reductases) was found to give the most reliable and consistent results for the gross identification of early myocardial infarction. Accordingly, the results will be interpreted according to difference in the intensity of the colour of the final reaction product of the enzymatic activity in the transversely cut heart slice. It must be emphasized that when staining with "non-specific dehydrogenase" is mentioned in the legends, NAD (coenzyme I) was always added to the incubating medium, unless otherwise stated.

Normal heart muscle stained homogeneously dark blue (Figure 4), variation in the intensity of the stain was considered a positive result and was diagnostic for identifying areas of early myocardial damage. Myocardial infarcts of stated clinical age, defined here as the time between the onset of acute myocardial infarction and death (under twenty-four hours), showed in the gross either focal diminution in dark blue diformazan staining with NBT or patches of red staining by the monoformazan of NBT (Figures 5, 6) and is referred to as a positive NBT test (negative enzymatic reaction). Myocardial infarcts of stated clinical age older than twenty-four hours showed circumscribed areas of near-absent staining (Figure 7), as did areas of laminar subendocardial fibrosis(Miller, Burchell and Edwards, 1951; Schwartz and Gerrity),

Figure 4: Nitroblue tetrazolium (NBT) non-specific dehydrogenase (left heart slice) and NADPH diaphorase (tetrazolium reductase, right heart slice) macrostaining of normal heart muscle.

Figure 5: Heart slice stained with non-specific dehydrogenase from a man of 65 years with a clinical history of less than one hour. Occlusion of the right coronary artery by a recent thrombus. Circumferential subendocardial diminution in the dark blue diformazan staining with NBT in the left ventricular wall. Posterior septum shows transmural diminution in staining.

Figure 6: Heart slice stained with B-hydroxybutyrate dehydrogenase from a man of 61 years with 4 hours clinical history. Occlusion of the left anterior descending and right coronary arteries by recent thromboses. Recent infarct of the left ventricular wall revealed by red staining with the monoformazan of NBT.

Figure 7: Heart slice stained with NADPH diaphorase from a man of 67 years with a clinical history of 27 hours. Occlusion of the right coronary artery by a recent thrombus. Transmural postero-septal infarct revealed by near absent staining of the lesion.

1975; Davies, Woolf and Robertson, 1976), including generalized ischaemia of the papillary muscles (Figure 8). Old scarred infarcts appeared as nearly white fibrous areas (Figure 9).

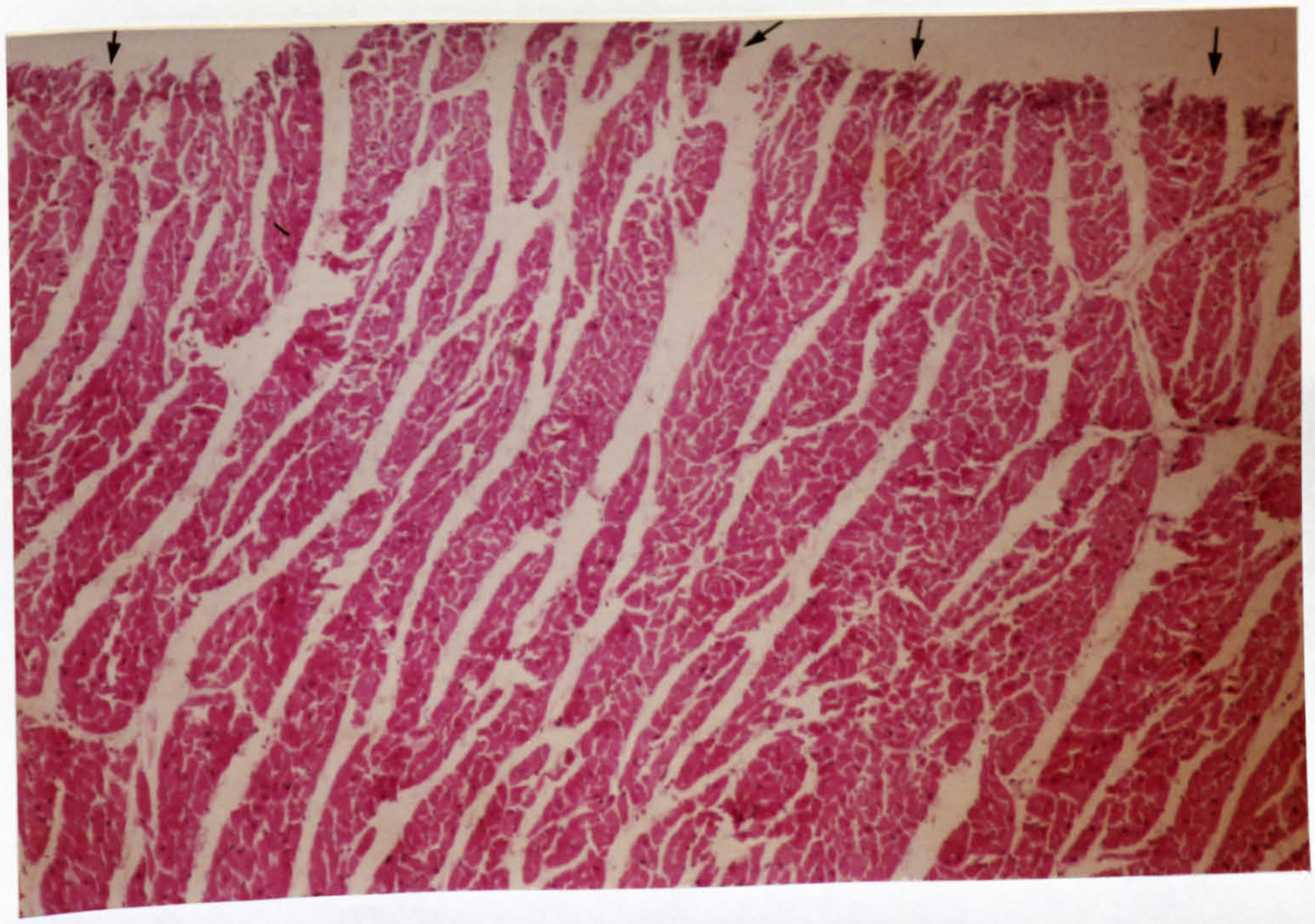
It was observed that there is a very narrow zone of about half mm wide immediately beneath the endocardium of the left ventricle, which stained dark blue in the otherwise faintly stained or non-stained infarcted area. This narrow zone represents viable cardiac muscle, presumably deriving its blood supply directly from the lumen of the left ventricle (Figure 10). This non-infarcted formazan-stained border, located in the immediate subendocardial position in the otherwise infarcted area, served as a built-in control for each heart slice.

The deposition of the formazan pigment is a surface phenomenon only, and it is not extracted from the tissue by the solvents used in the preparation of paraffin sections (Nachlas and Shnitka, 1963). After macroscopic staining with NBT, tissue blocks were cut at right angles to the plane of the slice and used for the preparation of paraffin-embedded sections; these were stained with haematoxylin and eosin, which permitted cross reference between the gross and microscopic findings. The formazan dye was discernible in the microscopic slides on the edges of the tissue (Figures 11A and B; 12A, B and C). In practice, a better histological preparation was obtained when paraffin sections were prepared from the non-enzymatically stained mirror-image surface of the infarct and the adjacent normal myocardium.

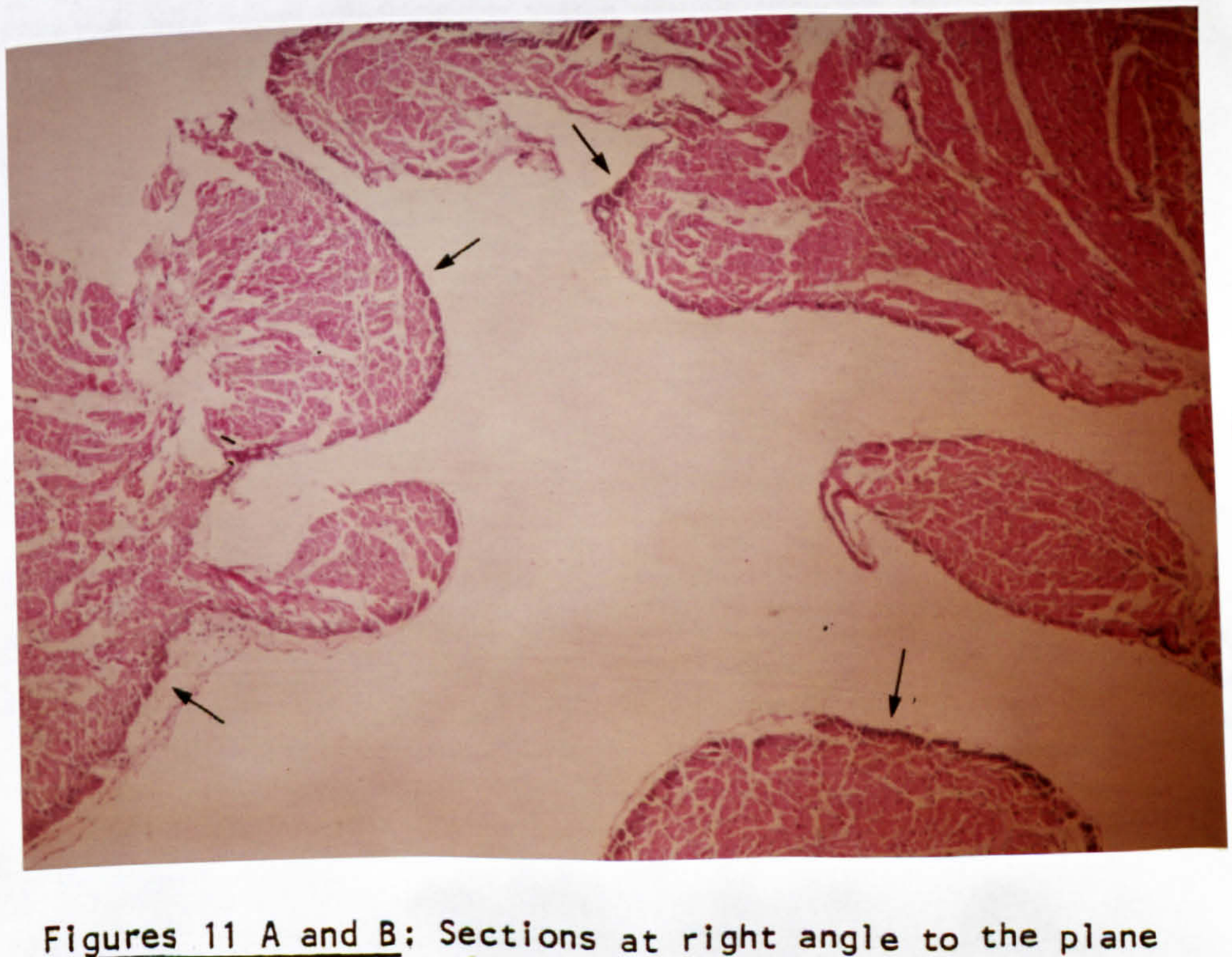
Figure 8: Heart slice stained with lactate dehydrogenase from a man of 70 years, showing laminar subendocardial fibrosis of the left ventricle. Triple coronary artery disease.

Figure 9: NADPH diaphorase macrostaining of a heart slice from a woman of 65 years. Old white fibrous scar in the posterior wall of the left ventricle. Recent zonal infarct of the septum; clinical history less than one hour. Occlusion of the right coronary artery by a recent thrombus.

Figure 10: Non-specific dehydrogenase macrostaining of a heart slice from a man of 70 years with a clinical history of 1½ hours. Occlusion of the right coronary artery by old and recent thromboses. Recent zonal infarction of the septum. Note the very narrow non infarcted (formazan-stained) zone immediately below the endocardium in the recently infarcted septum and in the fibrous shrunken scar of an old infarct in the posterior wall of both ventricles. Darkly stained viable fibres are seen in the recent and old infarcts.



A



B

Figures 11 A and B: Sections at right angle to the plane of a formazan stained heart slice. Dark blue formazan is visible on the surface of normal myocardial fibres (A, arrows), and of normal papillary muscles (B, arrows). Haematoxylin and eosin, X 125.

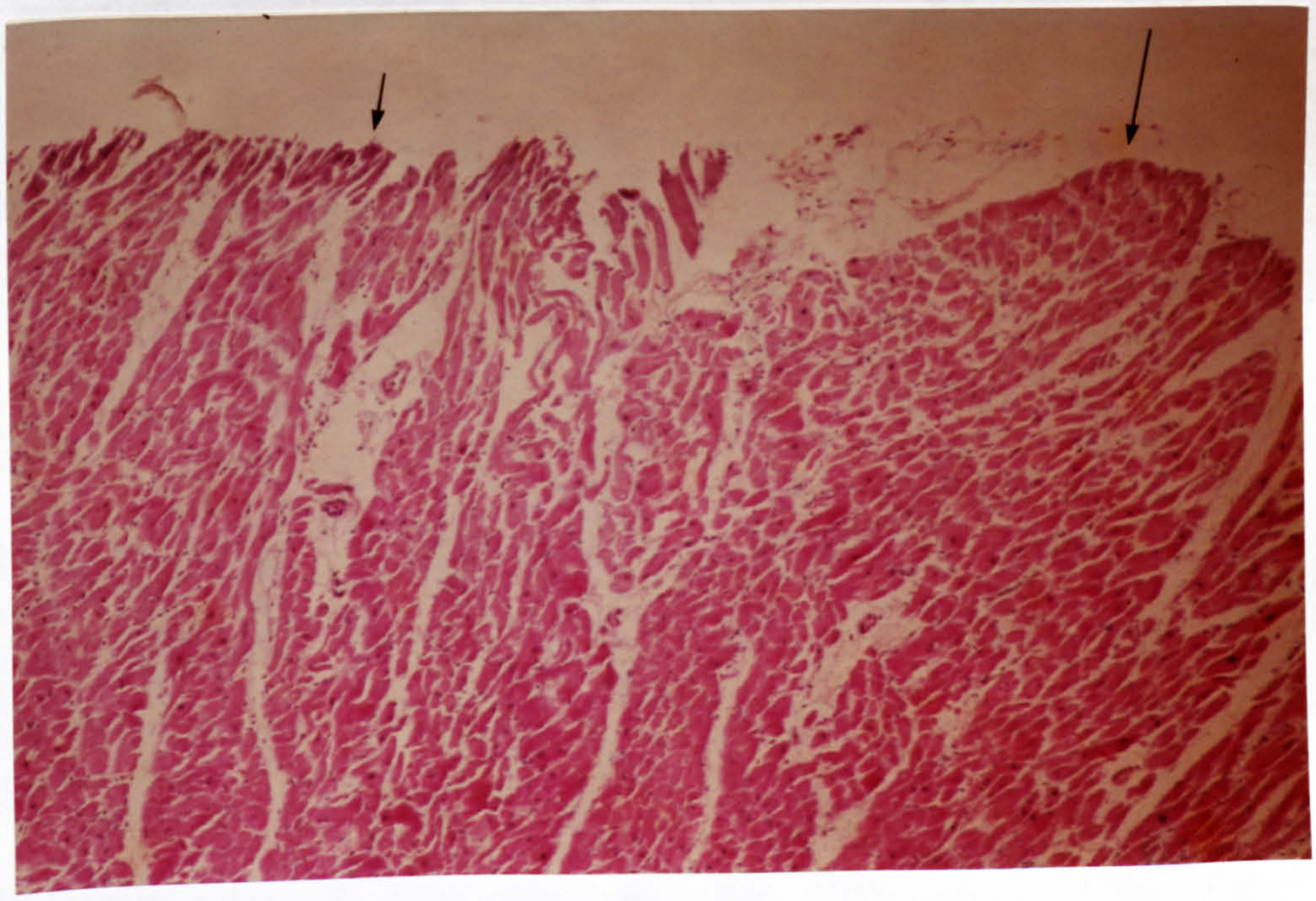
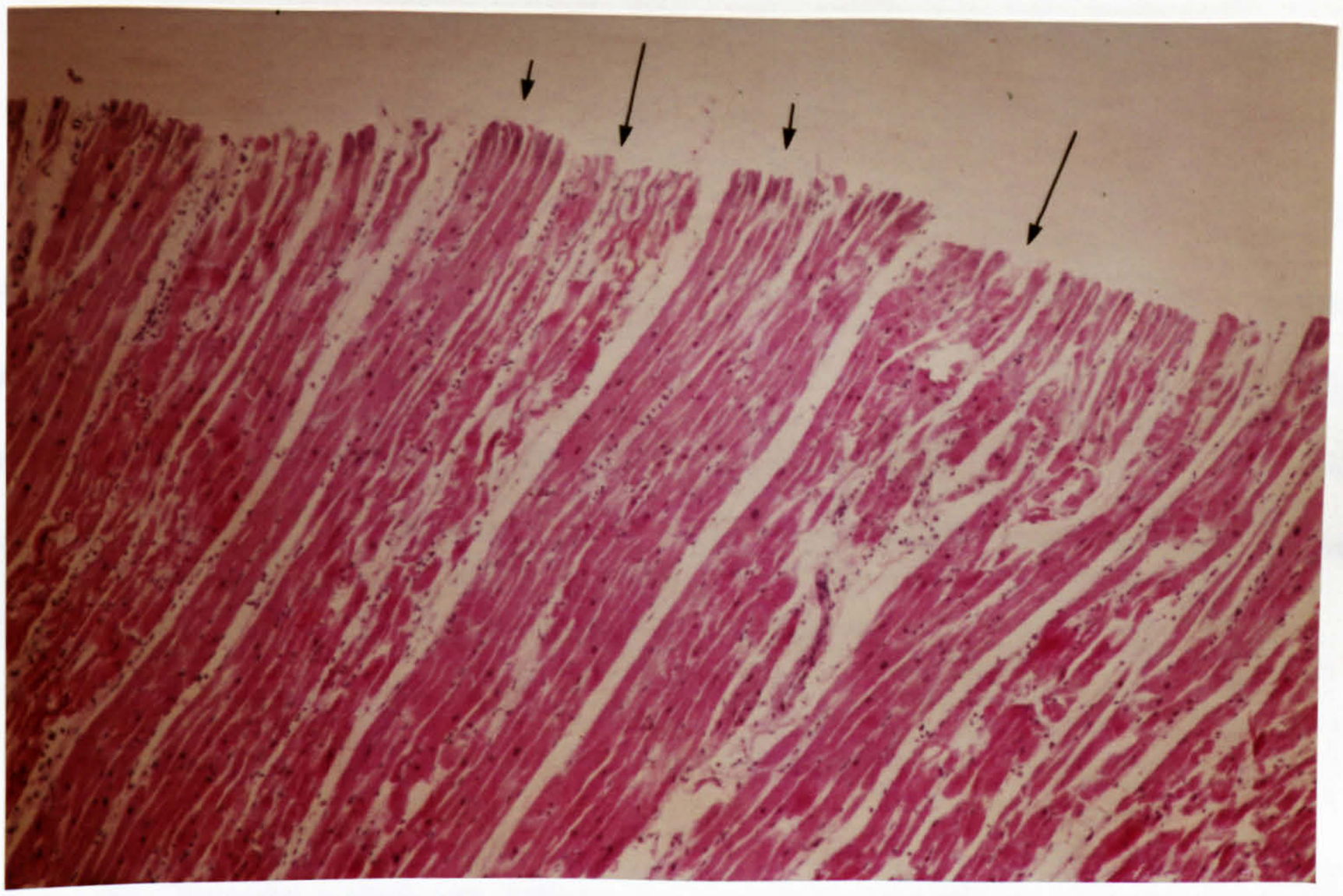


Figure 12 A: Section of heart muscle from a case with a clinical age under one hour. Dark blue formazan is visible on the surface of viable myocardial fibres (left, short arrow), and greatly diminished from the surface of the ischaemic fibres (right, long arrow). In this early stage there are no discernible histologic differences between the normal (formazan stained) and the ischaemic (nearly unstained). Haematoxylin and eosin, X 125.



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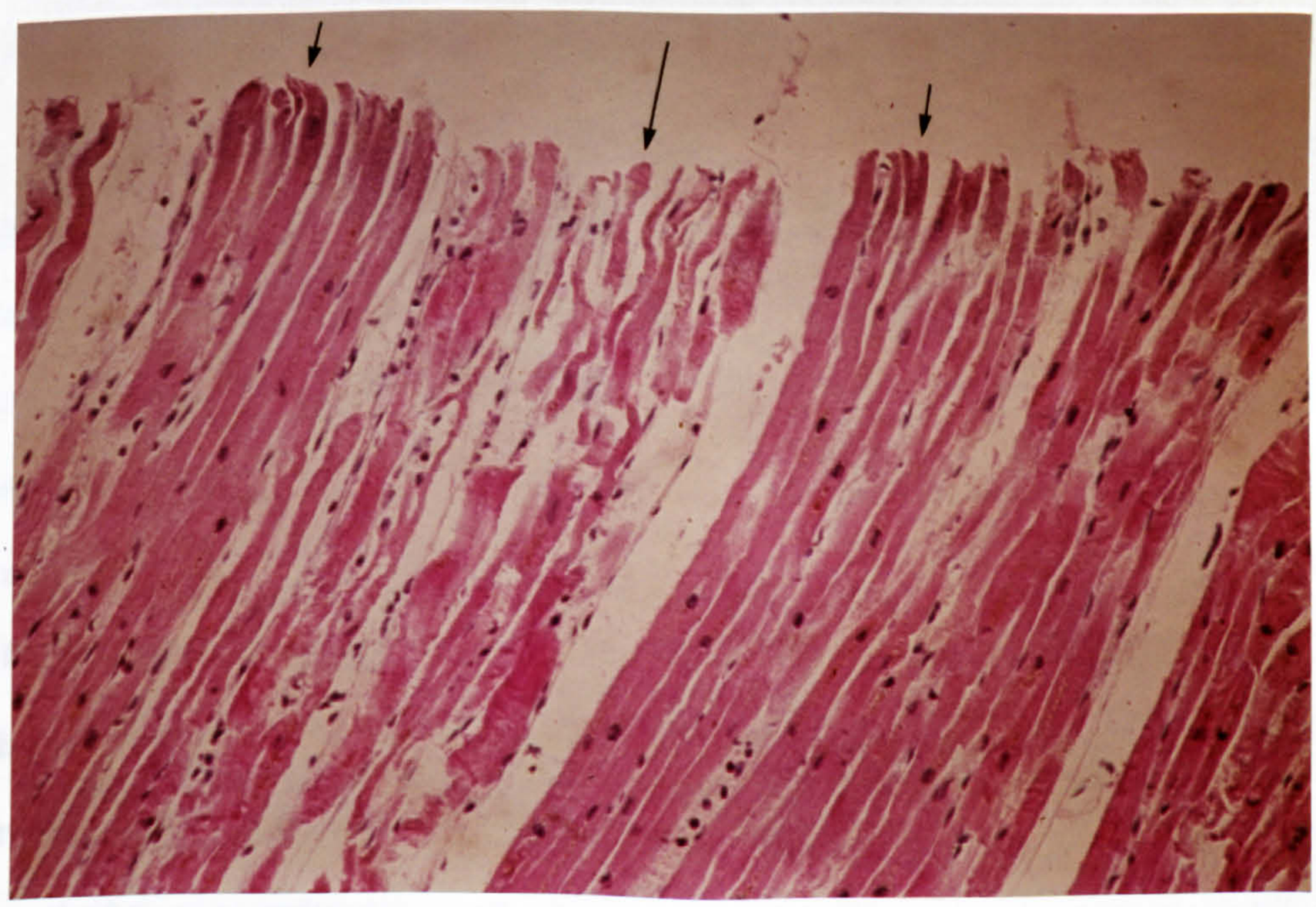


C

Figures 12 B and C: Heart muscle sections from a case with an 18 hour clinical history. Formazan pigment is seen on the surface of viable muscle fibres which look uniform (short arrows), and clearly absent from the surface of the well-defined infarcted fibres (long arrows). These last are characterized by eosinophilia, a swollen hyaline appearance, distoriton and neutrophil polymorph infiltration. Haematoxylin and eosin, X 125 (Fig. 13 B); X 232 (Fig. 13 C).

B. Anatomical localisation of myocardial infarction

In the localisation of infarcts in the transverse heart slice, the left ventricle was considered to be divided into three parts. These parts were designated anterior, posterior and lateral. Figure 13 demonstrates the anatomical localisation of myocardial infarction when the ventricles were sliced into serial sections transverse to the long axis of the heart from the apex to the base.

C. Topographical pattern of myocardial infarction

The macroscopic pattern of myocardial infarction was recognised as set out by Davies (1977). Infarcts involving all or almost all of the entire thickness of a portion of the left ventricular wall were classified as "transmural", while infarcts involving not more than the endocardial half of the thickness of the wall of the left ventricle were classified as subendocardial infarcts. Two types of subendocardial infarcts were recognised. Infarction which involved only the subendocardial zone of a segment of the ventricular wall of the left ventricle were termed "zonal", while infarcts involving predominantly the whole circumference of the subendocardial zone of the left ventricle - including the centres of the papillary muscles - were termed "laminar" or "circumferential" infarcts. A combination of any of these patterns of myocardial infarction was referred to as a "mixed" infarct. It was also observed that the diminution in the intensity of staining with the NBT was sometimes localised in the papillary muscles only. This was characteristic of a well-known condition which may be termed isolated papillary muscle infarction or focal ischaemia (Figure 14).

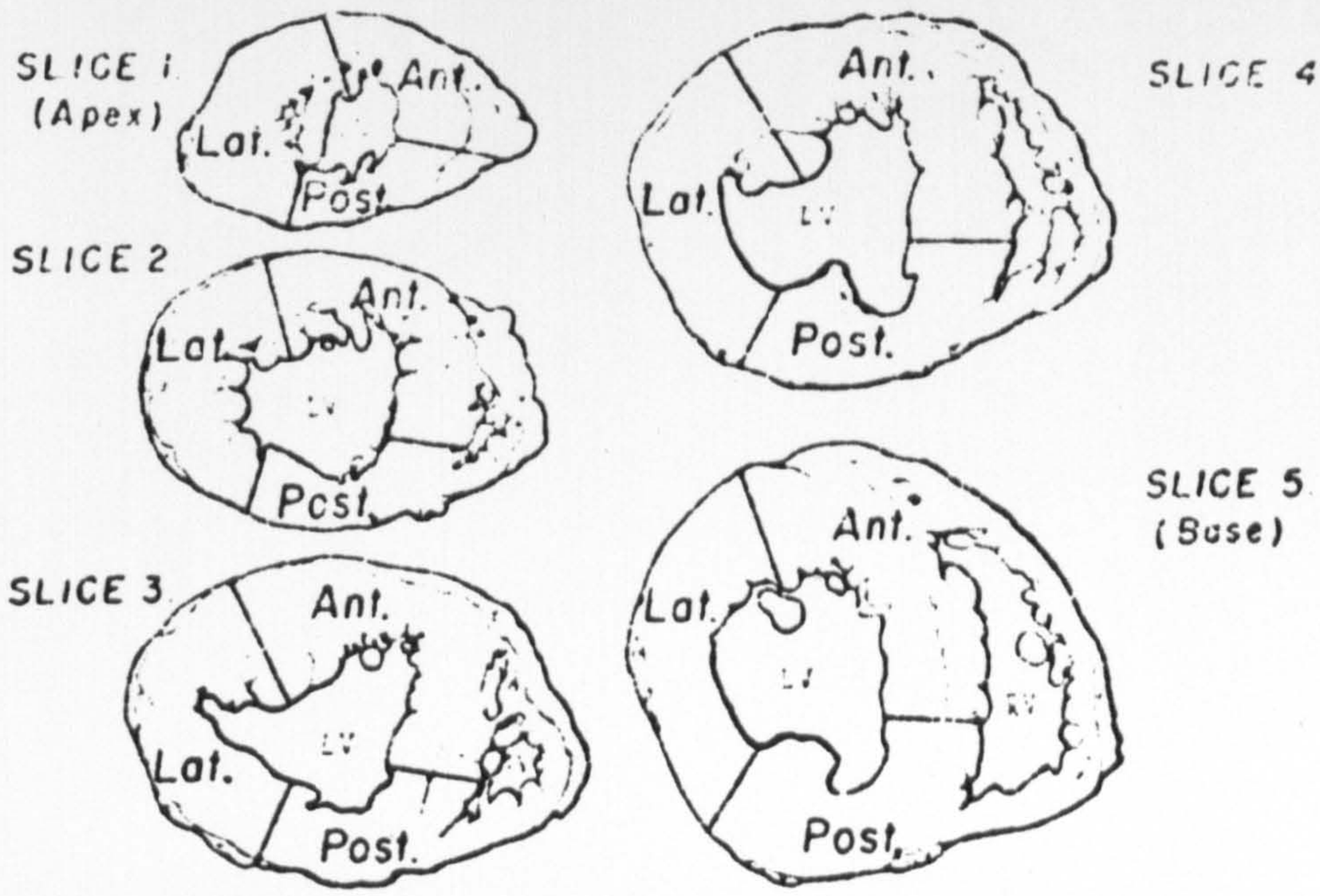


Figure 13: Anatomical localization of myocardial infarction.

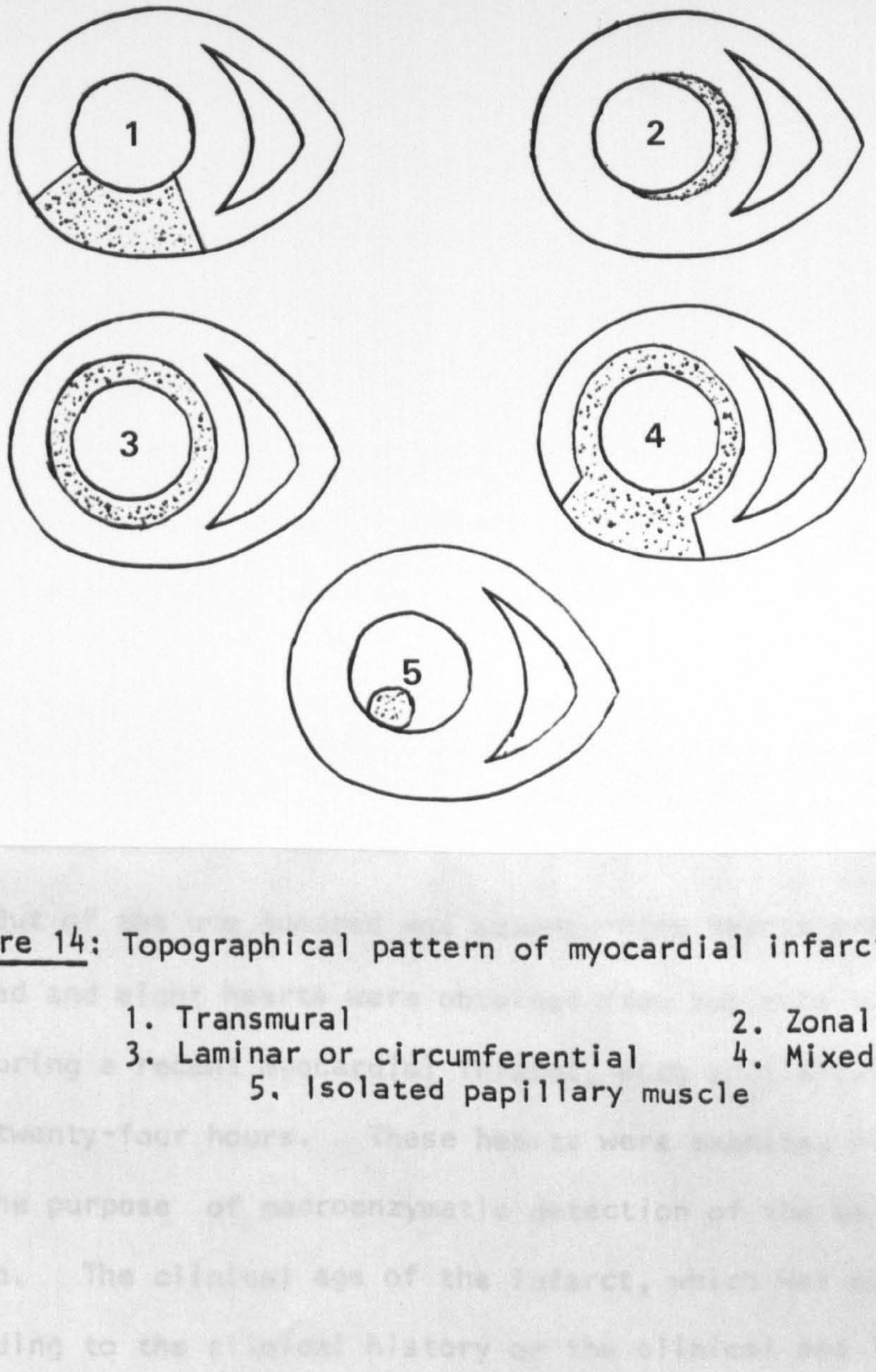


Figure 14: Topographical pattern of myocardial infarction.

1. Transmural
2. Zonal
3. Laminar or circumferential
4. Mixed
5. Isolated papillary muscle

D. Criteria for histological dating of myocardial infarction

The microscopical criteria for dating myocardial infarction as set out by Mallory, White and Salcedo-Salgar (1939); Lodge-Patch (1951), and Olsen (1973), is presented in Table 17, and was classified into four histological grades (A, B, C and D). Grade A comprised apparently normal myocardial fibres and normal stroma. Oedema, swollen hyaline appearance to fibres, distortion and some loss of internal structure were features of infarction from about five hours after coronary artery occlusion and these were included in Grade B. Increased eosinophilia of myocardial fibres also appear at this time and was a particularly valuable and readily detectable sign. Grade C was recognised by polymorphnuclear infiltration and was a feature of infarction from about twelve hours, while clear necrosis and absence of nuclei were considered histological features of infarction older than twenty-four hours and were considered in Grade D.

II. MACROENZYMATIC IDENTIFICATION OF EARLY MYOCARDIAL INFARCTION

Out of the one hundred and seventy-nine hearts examined, one hundred and eight hearts were obtained from subjects suspected of harbouring a recent myocardial infarct, with a clinical age of less than twenty-four hours. These hearts were examined histochemically for the purpose of macroenzymatic detection of the early myocardial lesion. The clinical age of the infarct, which was estimated according to the clinical history or the clinical and laboratory diagnosis or both and the mode of death, was classified into four

Table 17. Criteria for histological dating of myocardial infarcts

Grade	Microscopy	Time
A	Presumptive; no eosinophilia; no loss of internal structure	up to 5 hours*
B	Eosinophilia; swollen hyaline appearance to fibres; distortion and some loss of internal structure	5 hours onwards* †
C	Neutrophil polymorph infiltration (more than margination and local perivascular infiltration) plus B	12-24 hours onwards* † + +
D	Clear necrosis and absence of nuclei, plus B and C	1 - 2 days onwards † + +

* Mallory, White and Salcedo-Salgar (1939)

† Olsen (1973)

\+ Lodge-Patch (1951)

\+

clinical age groups (Table 6, page 64). It is accepted here that in many cases the clinical age was shorter than the true pathological age and indeed, this was shown to be so in some cases (see below).

A. Suspected myocardial infarction of estimated clinical age under one hour

Out of the forty-eight suspected cases of recent myocardial infarction examined in this group, the nitroblue tetrazolium (NBT) test was positive (negative enzymatic reaction) in twenty-seven cases, and was negative (enzymatically stained) in the remaining twenty-one cases (Table 18).

In these twenty-seven NBT-positive "under one hour" cases, myocardial infarction was revealed in twenty-three only after myocardial staining with nitroblue tetrazolium (Figures 15, 16, 17, 18, 19, 20). In these twenty-three hearts, the infarct had not been suspected or recognised during ordinary macroscopic evaluation. Subsequent microscopic examination based on the histological criteria listed in Table 17 demonstrated pre-inflammatory signs (Grade B; Figure 21) in six cases, while histologic evidence of the lesion was absent (Grade A; Figure 22) in seventeen cases. It could, of course, be argued that these seventeen cases were artefactual false - positives. However, no false positives were noted in group with a clinical age group greater than twelve hours, so in arguing by analogy it is unlikely that the seventeen cases reflected an artefact.

Table 18. Nitroblue tetrazolium screening of myocardial infarcts of estimated clinical age under one hour

NBT Macro-reaction	Number of cases (Total 48)	Naked eye infarction				Coronary artery thrombosis
		A	B	C	D	
		Number of cases				
Transmural	10	3	5	3(1)	2(2)	7
Zonal	14	1	10	4(1)		13
Laminar	1	0		1		0
Papillary muscle	1	0	1			1
Mixed	1	0	1			1
Total	27	4	17	8(2)	2(2)	22
Negative	21	2	21			19

The number between brackets represents the number of cases with naked eye infarction at autopsy.

Figure 15: Two opposing heart slices from a 48-year old man with a clinical history of less than one hour. Enzymatic macrostaining with non-specific dehydrogenase revealed circumferential subendocardial recent infarction of the left ventricular wall. No naked eye evidence of infarction in the adjoining unstained slice (right). Occlusion of the left anterior descending and right coronary arteries by recent thromboses.

Figure 16: Two opposing heart slices from a 65-year old man with a clinical history of less than one hour. Non-specific dehydrogenase macroreaction (left slice) revealed a recent mixed infarct of the left ventricle, which was not recognised in the unstained slice (right). Occlusion of the right coronary artery by a recent thrombus.

Figure 17: Non-specific dehydrogenase macrostaining of a heart slice from a 60-year old woman with a clinical history of less than one hour. Recent postero-septal transmural-mottled infarct of the left ventricle and of the posterior papillary muscle. Occlusion of the right coronary artery by a recent thrombus.

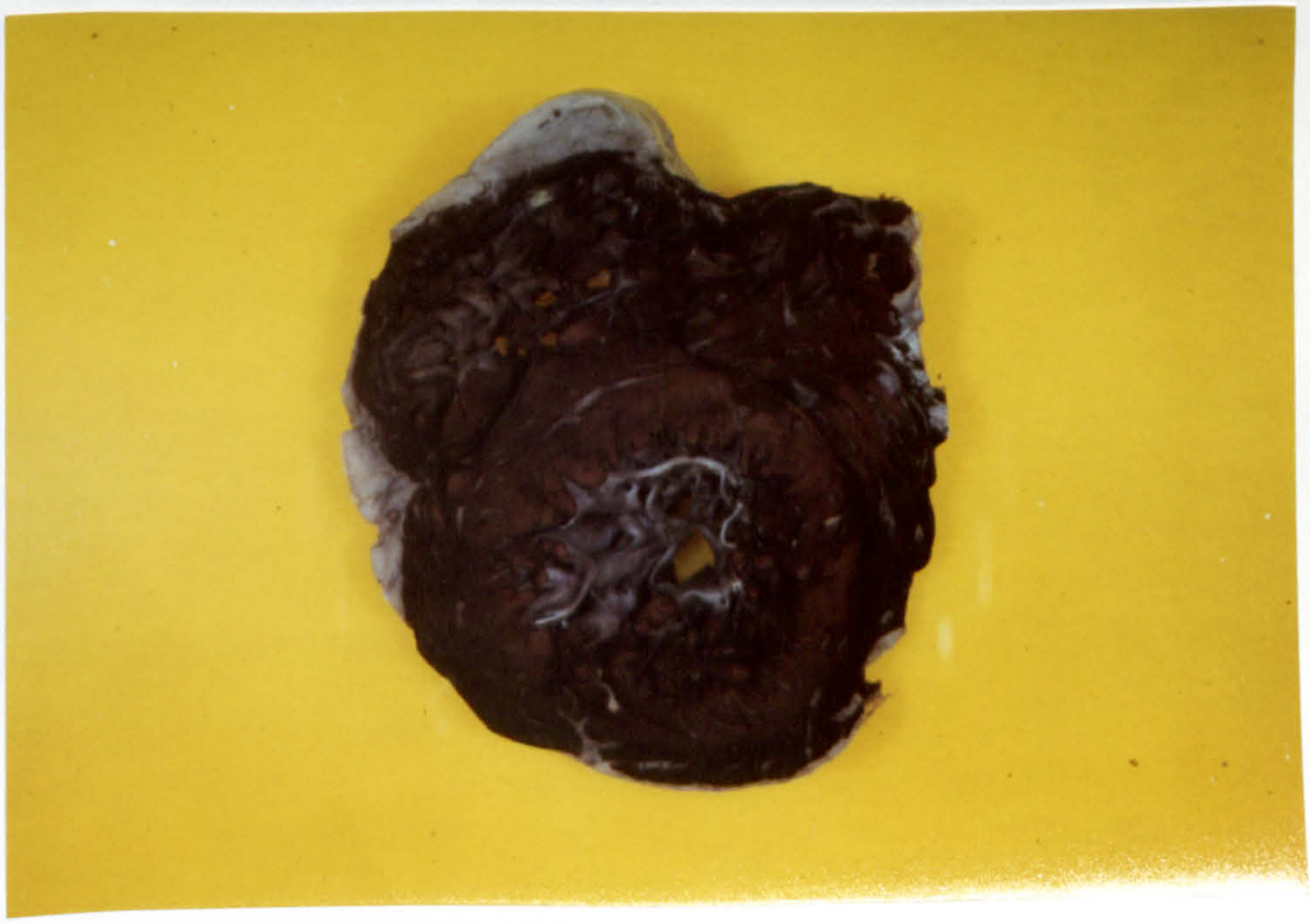


Figure 18: NADH diaphorase macrostaining of a heart slice from a 55-year old man with a clinical history of less than one hour. Recent mixed infarct of the left ventricular wall. Occlusion of the right coronary artery by a recent thrombus.

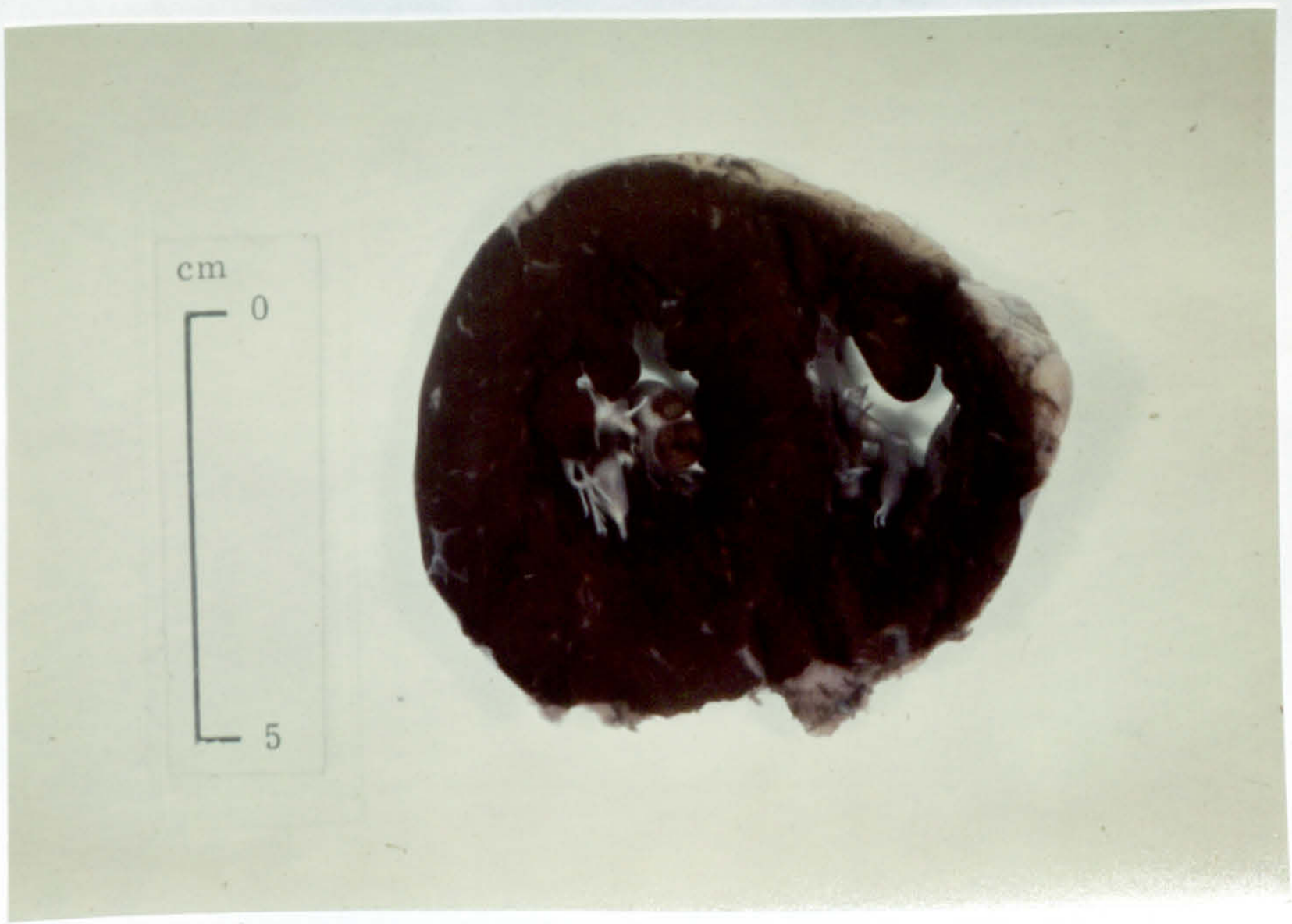


Figure 19. B-hydroxybutyrate dehydrogenase macrostaining of a heart slice from a 50 year old woman, with a clinical history of less than one hour, revealing isolated posterior papillary muscle infarct. Occlusion of the right coronary artery by a recent thrombus.

A

B

Figure 20: Non-specific dehydrogenase macrostaining of a heart slice from a 63-year old man with a clinical history of less than one hour. Recent postero-septal infarct revealed by the enzymatic reaction in slice A (arrows), and its other surface B (arrows). A shows an old antero-septal infarct, while B shows additional coarse fibrosis of the posterior wall presumably a result of previous zonal infarct in this region. Occlusive atheroma of the right coronary artery; recanalised left anterior descending coronary artery.

Figure 21: Section from infarcted area of the heart of a man of 60 years with a clinical history of less than one hour. Swelling and distortion of some myocardial fibres and interstitial oedema. Grade B histology (5 - 12 hours). Underestimate of the clinical age of the infarct. Haematoxylin and eosin, X 500.

Figure 22: Section of infarcted area of the heart in a man of 55 years with a clinical history of less than one hour. Normal appearance to myocardium. Grade A histology. Haematoxylin and eosin, X 500.

Four hearts in this clinical age group of under one hour showed naked eye evidence of recent myocardial damage at autopsy as areas of congestion and slight discolouration. The NBT test was positive in these cases, and helped to demonstrate the extent of the infarct by delineating its boundaries. The increase in contrast between viable and necrotic myocardium afforded by the stain made the delineation of the infarcts much more precise (Figure 23). In these four hearts the histologic findings were also more consistent with an infarction process older than the estimated clinical age. Histologic examination showed eosinophilic swollen myocardial fibres and oedema in two hearts (Grade B; Figure 24), and neutrophil polymorphnuclear infiltration (Grade C; Figure 25) in two hearts.

The major branches of the coronary arteries of these twenty-seven NBT-positive hearts showed occlusive thromboses in twenty-two cases, and severe stenosis due to coronary artery atherosclerosis in the five other cases.

Figure 23: Two opposing heart slices from a 42-year old man with a clinical history of less than one hour. Occlusion of the left anterior descending coronary artery by a recent thrombus. Unstained heart slice (left) showed slight pallor of the septum. The lesion was identified with certainty and was better delineated after the NBT enzymatic macroreaction (right slice).

Figure 24: Section of infarcted area in heart of a man of 65 years with a clinical history of under one hour. Eosinophilic swollen appearance to some myocardial fibres and interstitial oedema. Grade B histology.
Haematoxylin and eosin, X 500.

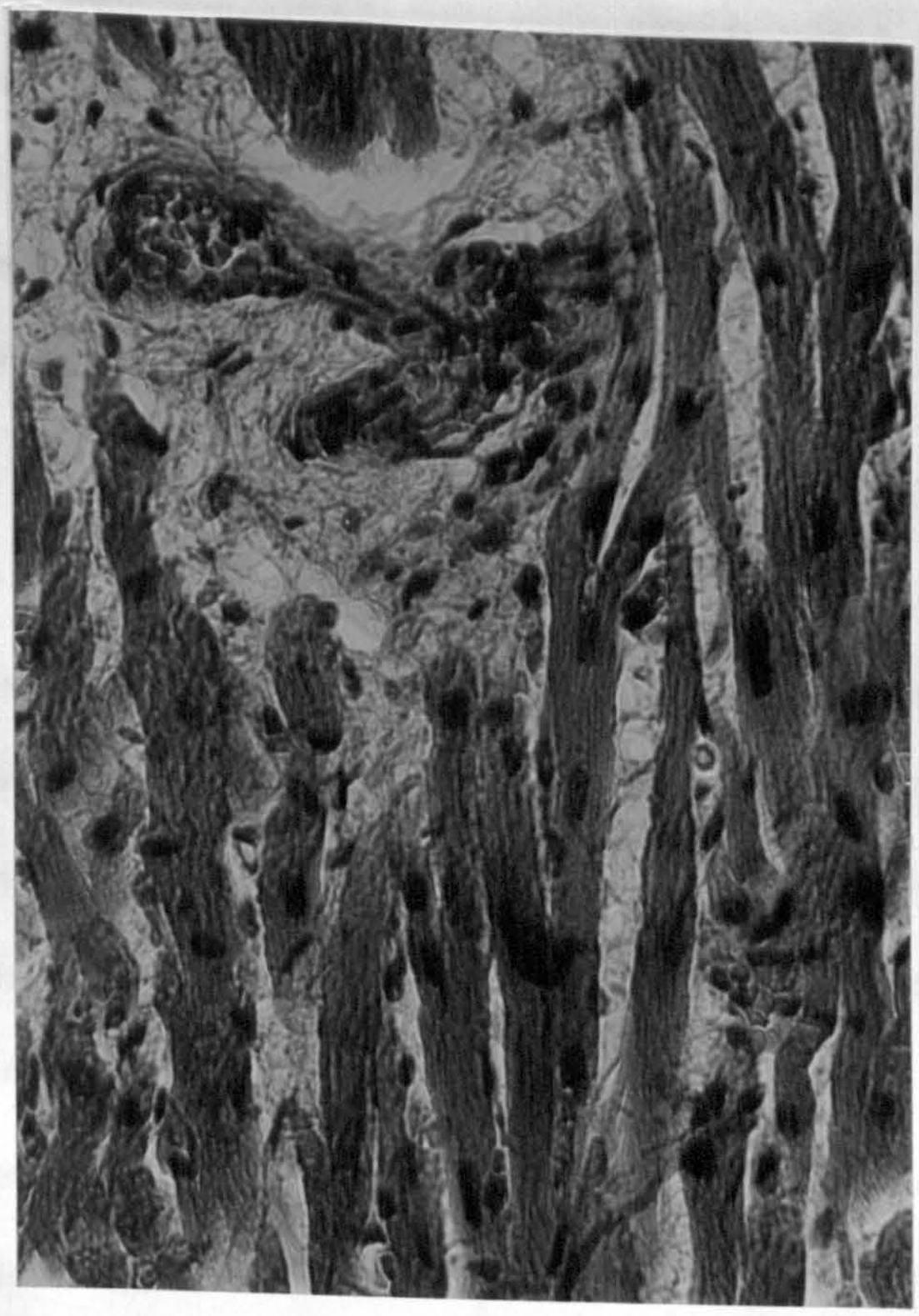


Figure 25: Section of infarcted area in the heart of a man of 70 years with a clinical history of under one hour. Swollen disorganized myocardial fibres and neutrophil polymorph infiltration of the myocardium. Grade C histology. Haematoxylin and eosin, X 500.

In twenty-one cases of suspected myocardial infarction of clinical age under one hour, the macroenzymatic examination with NBT did not reveal an infarct. There was no naked eye evidence of myocardial infarction at autopsy in these hearts, apart from two hearts which showed equivocal pallor in the myocardium in one, and haemorrhagic areas in the other. Histologically they were all of Grade A. Coronary arteries examined at autopsy revealed thrombotic occlusion in nineteen cases, and severe coronary stenosis in two cases.

The naked eye appearance of myocardial infarction in two of the twenty-one NBT-negative hearts did not correspond to the anticipated area of myocardium supplied by the occluded coronary artery. The first case was of a man aged sixty-two years who was treated in the hospital from carcinoma of the prostate. There was no previous history or clinical evidence of ischaemic heart disease. He suddenly collapsed, and attempts at resuscitation failed, including external cardiac massage, and the patient died some twenty minutes later. Autopsy examination performed thirty-six hours after death revealed fresh thrombotic occlusion of the first two cm of the left anterior descending coronary artery, and moderate atheroma of the right and left circumflex coronary arteries. An area of congestion was seen at the junction of the posterior wall of the left ventricle and of the posterior septum which showed a positive NBT staining reaction and Grade A microscopy.

The second case was of a man aged forty-eight years who collapsed at work and died suddenly. Necropsy performed eighteen hours after death revealed a fresh thrombotic occlusion of the proximal part of the left circumflex coronary artery, and mild atheromatous changes in

both the left anterior descending and right coronary arteries. Equivocal pallor was seen in the anterior wall of the left ventricle, but it stained blue with NBT. On histological examination of the mirror-image surface, it showed apparently normal myocardial fibres and stroma.

B. Suspected myocardial infarction of estimated clinical age one to five hours

The hearts from twenty-eight cases of suspected recent myocardial infarction were examined in this group. The result of the macrostaining with nitroblue tetrazolium is shown in Table 19. The NBT test was positive (negative enzymatic reaction) and, thus, diagnostic of recent myocardial damage in twenty-six cases, and was negative (positive enzymatic reaction) in the remaining two cases.

Out of the twenty-six hearts in which the NBT test was positive, the gross necropsy changes that are characteristic of recent myocardial infarction were absent in twenty-two cases, and the lesion was only recognised after staining with NBT (Figures 26, 27, 28). Histological evidence of recent myocardial damage was absent in fourteen cases (Grade A), while the pre-inflammatory microscopic signs (Grade B) were demonstrated in nine cases. Neutrophil polymorphnuclear infiltration was seen in two cases (Grade C), and one case exhibited clear necrosis (Grade D).

Table 19. Nitroblue tetrazolium staining of myocardial infarcts of estimated clinical age one to five hours.

NBT Macro-reaction	Number of cases (Total 28)	Naked eye infarct	Microscopic grade			Coronary artery thrombosis
			A	B	C	D	
		Number of cases					
Transmural	13	2	7	4	1*	1*	10
Zonal	8	1	5	2	1*		6
Laminar	2	1	1	1*			0
Papillary muscle	0	0					0
Mixed	3	0	1	2			3
Total	26	4	14	9	2	1	19
Negative	2	0	2				2

* with naked eye infarct.

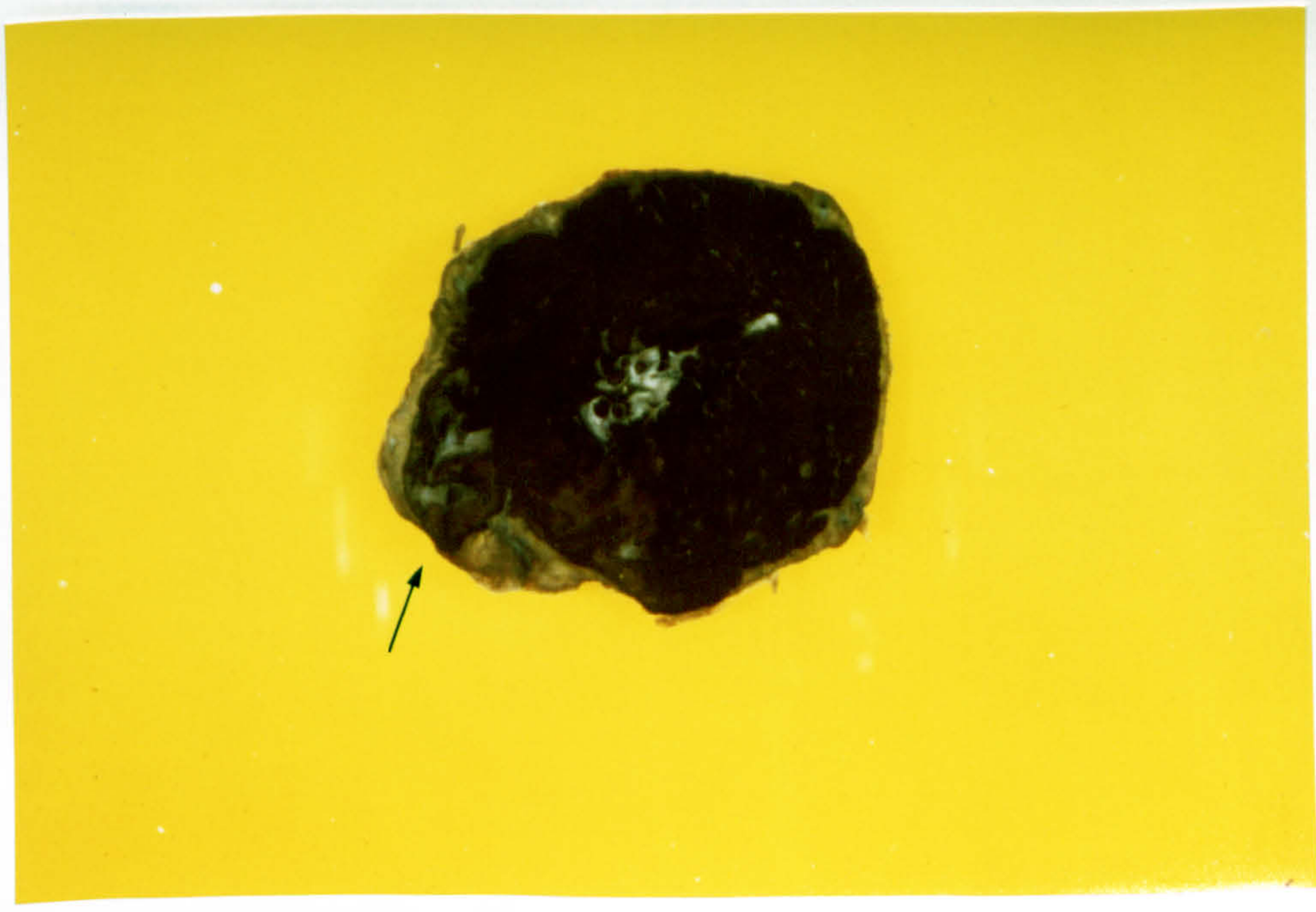


Figure 26: β-hydroxybutyrate dehydrogenase macrostaining of a heart slice from a man of 58 years with a 1½ hour clinical history. Recent transmural infarct of the posterior wall of the left ventricle and right ventricle (arrow). Occlusion of the right coronary artery by a recent thrombus.

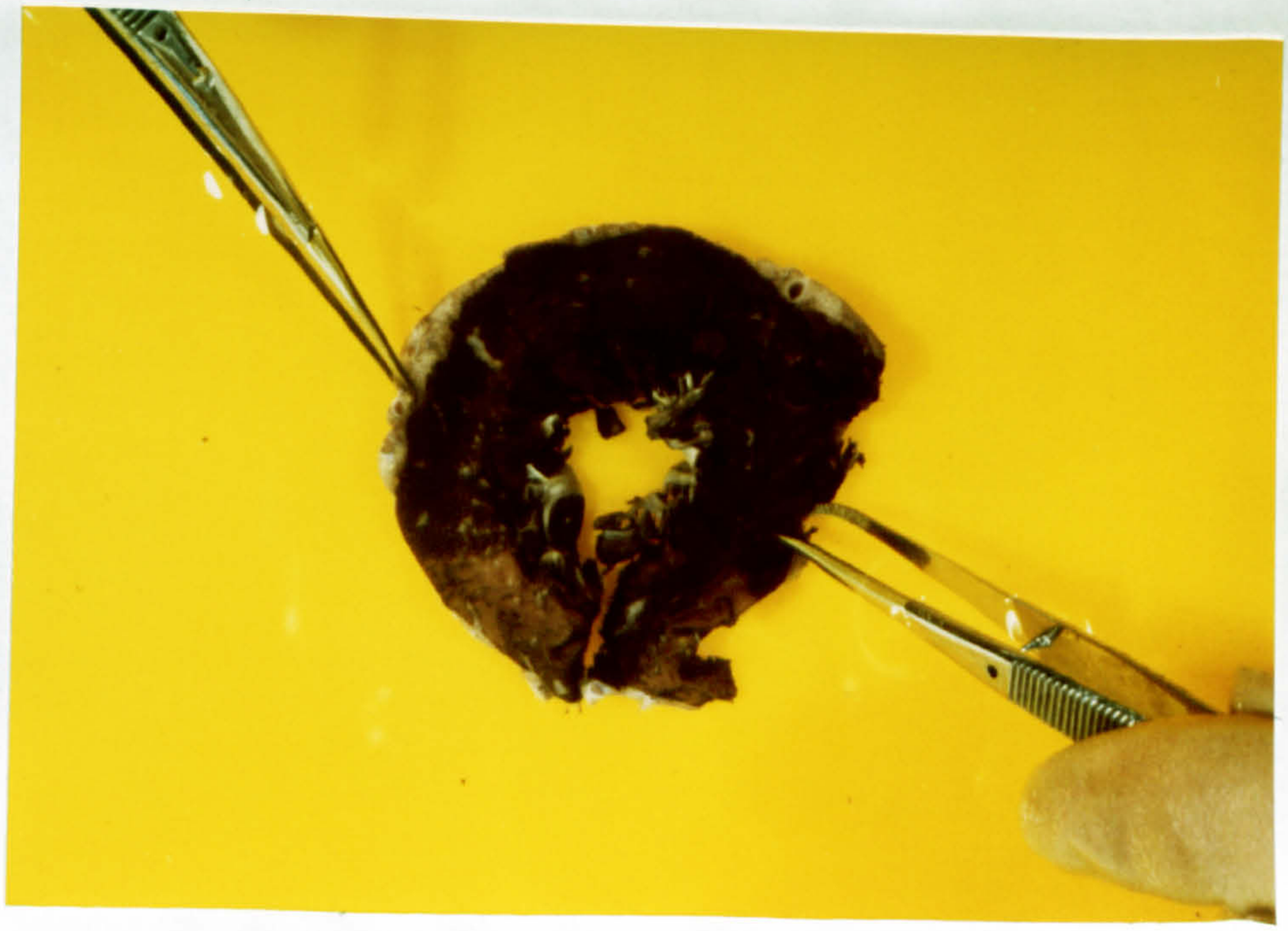


Figure 27: NADH diaphorase macrostaining of a heart slice from a man of 62 years with a 3 hour clinical history. Occlusion of the right coronary artery by a recent thrombus. Recent postero-septal transmural infarct of the left and right ventricles. Some viable (enzymatically stained) fibres are seen in the infarcted area.

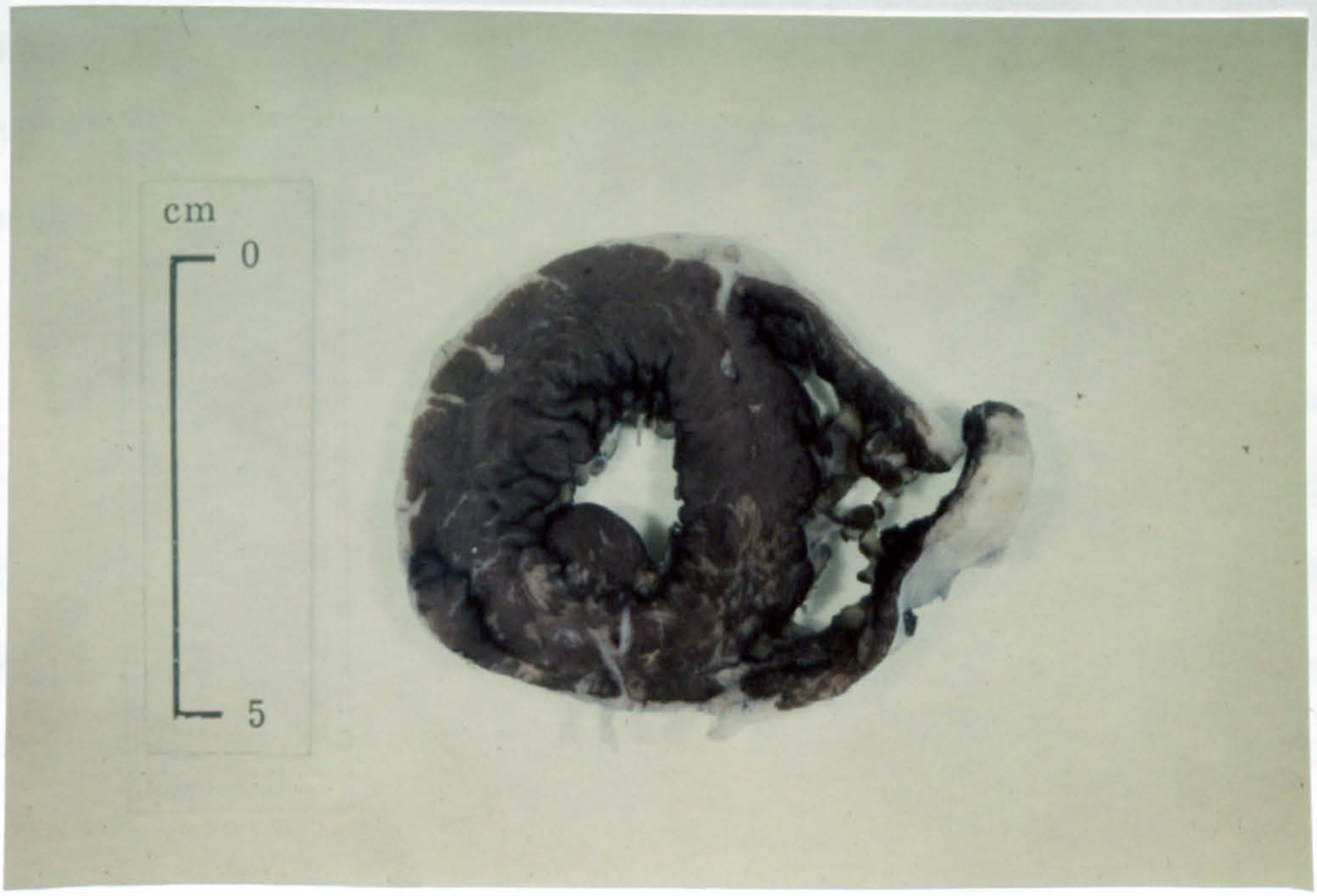


Figure 28. Non-specific dehydrogenase macrostaining of a heart slice from a man of 26 years with a 4½ hour clinical history. Recent postero-septal transmural infarct of the left and right ventricles. Occlusion of the right coronary artery by a recent thrombus.

Naked eye infarction was recognised at autopsy in four of the twenty-six NBT-positive hearts, which was also confirmed on microscopic examination. In these hearts the NBT test was also positive and it well illustrated the topography of the myocardial lesion. One of these four cases was of a man aged thirty-two years who attended casualty department complaining of pain in the chest of two hours duration. There was no previous history of chest pain. He died thirty minutes later. Autopsy examination performed twenty-four hours after death revealed recent thrombotic occlusion of the right coronary artery two cms from its origin. The myocardium of the right posterior wall of the left ventricle was soft and showed a pale area surrounded by a haemorrhagic border. The nitroblue tetrazolium macroreaction revealed a postero-septal transmural infarction (Figure 29), and microscopic examination of the mirror-image surface showed neutrophil polymorph infiltration (Figure 30). Although the estimated clinical age in this case was two and a half hours, both the gross and the microscopic appearances of the damaged myocardium were more consistent with an infarct duration of about twenty-four to forty-eight hours. It is likely that the infarct in this case remained painless or silent for some time before manifesting itself clinically. The same as with the other three cases in which the estimated clinical age did not coincide with the gross and histologic findings.

Examination of the coronary arteries in the twenty-six NBT-positive hearts showed recent coronary artery thrombosis in nineteen cases, and severe atherosclerotic coronary artery stenosis in four cases. Two other hearts were from patients who died in the operating theatre. The first of these two cases was a seventy-year old male patient who was undergoing

Figure 29: Lactate dehydrogenase macrostaining of a heart slice from a man of 32 years with a clinical history of 2½ hours. Occlusion of the right coronary artery by a recent thrombus. Recent postero-septal transmural infarct of the left and right ventricles. The lesion was recognised at autopsy (silent infarction).

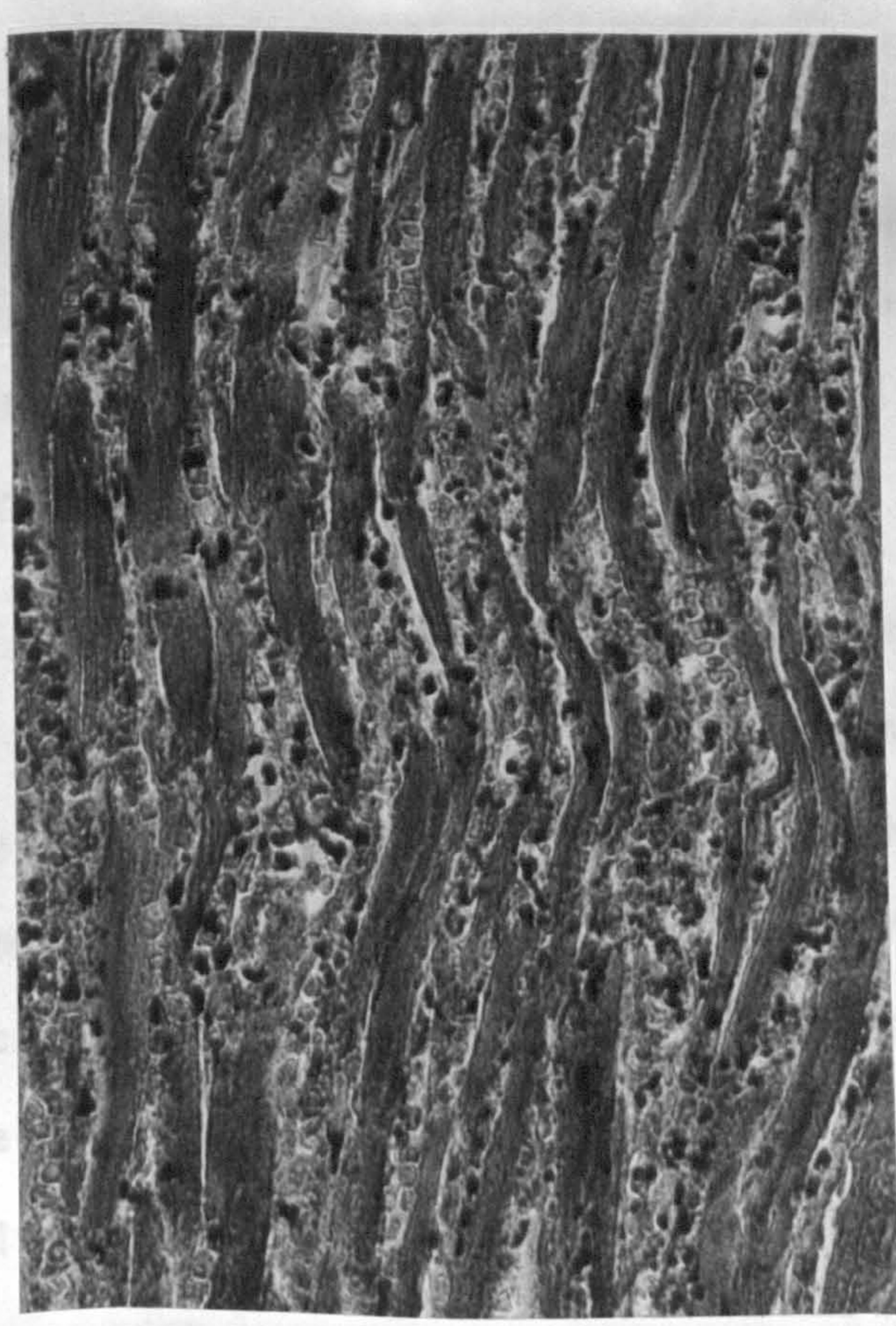


Figure 30: Section of infarcted area of heart in a man of 32 years with a clinical history of 2½ hours. Hyalinization of muscle fibres, nuclear pyknosis, and structural disorganisation. Neutrophil polymorph infiltration. Grade D histology. A case of silent infarction. Haematoxylin and eosin, X 310.

open cardiac surgery and died in the operating theatre two hours from the beginning of the operation. Autopsy examination performed seven hours after death showed old thrombotic occlusion of the left circumflex coronary artery, moderate atheroma of the left anterior descending coronary artery and of the right coronary artery. There was patchy fibrosis of the lateral wall of the left ventricle. Enzymatic macrostaining with NBT revealed a circumferential negative enzymatic reaction (positive result) in the left ventricular wall (Figure 31), which appeared otherwise normal at necropsy and was of Grade A histology.

The second case was of a sixty-three year old male patient who was undergoing surgical treatment for a ruptured atheromatous aneurysm of the abdominal aorta and died in the operating theatre four hours later. Necropsy performed five hours after death showed coarse scarring of the left ventricle of the heart and advanced occlusion of the coronary arteries by atheroma. The macroscopic dehydrogenase reaction was circumferentially positive in the left ventricular wall (Figure 32), and this was consistent with a recent myocardial damage. The lesion was not apparent at necropsy and histologically it was of Grade B.

In another heart, autopsy showed a recent antero-septal myocardial infarct with rupture of the interventricular septum. Examination of the coronary arteries in this case showed thrombotic occlusion of the left anterior descending coronary artery four cm from its origin and ectasia of the right coronary artery. The NBT enzymatic macroreaction was clearly absent (positive result) in the antero-septal wall of the left ventricle, as well as in the posterior wall of the left ventricle (Figure 33), a feature which is consistent with recent myocardial damage to the posterior ventricular wall. The lesion was not suspected or recognised at autopsy, and histologically it was of Grade A.

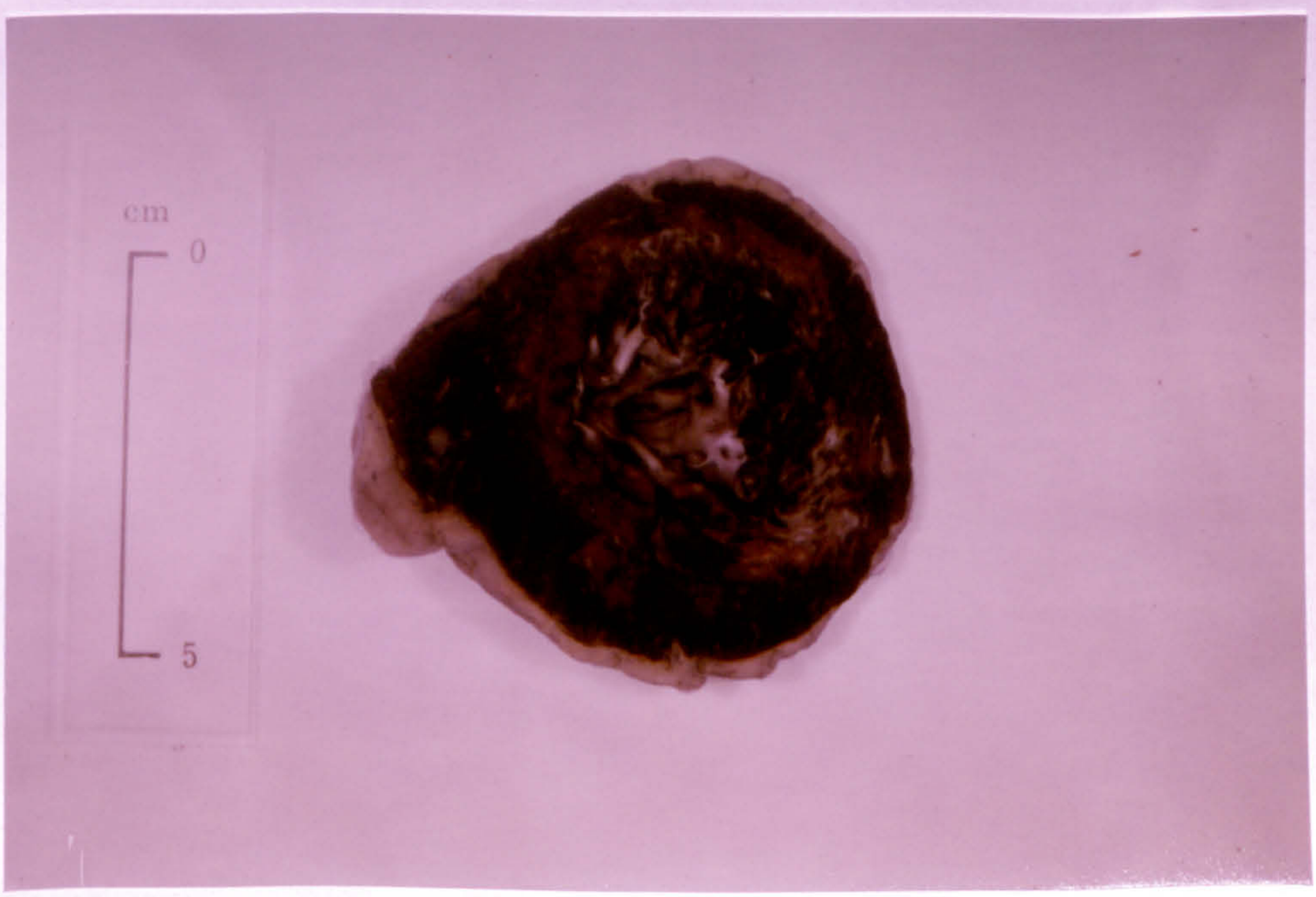


Figure 31: NADH tetrazolium reductase macrostaining of a heart slice from a man of 70 years who was undergoing open cardiac surgery and died in the operating theatre 2 hours from the time of operation. Recent circumferential myocardial necrosis of the left ventricular wall. Old thrombotic occlusion of the left circumflex coronary artery; moderate atheroma of the left anterior descending and right coronary arteries.

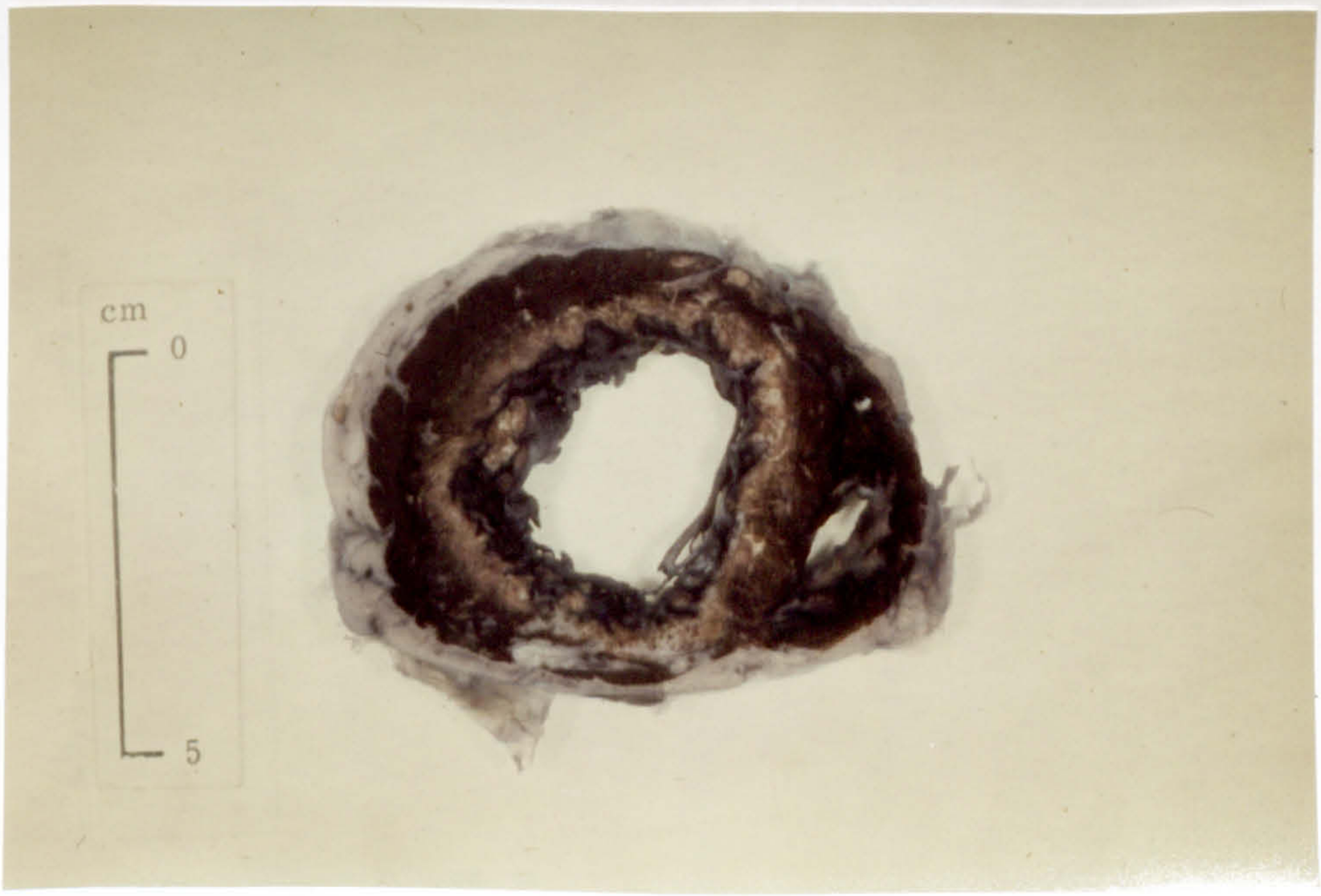


Figure 32: Non-specific dehydrogenase macrostaining of a heart slice from a man of 63 years who was undergoing surgical treatment of a ruptured aneurysm of the abdominal aorta and died 4 hours from the time of operation. Recent laminar necrosis of the left ventricular wall. Coarse fibrosis of the posterior wall of the left ventricle. Atheromatous stenosis of coronary arteries.

Figure 33: β-hydroxybutyrate dehydrogenase macrostaining of a heart slice from a woman of 84 years. Thrombotic occlusion of the left anterior descending coronary artery, and ectasia of the right coronary artery. Recent antero-septal infarct with rupture of the inter-ventricular septum (arrow). Recent zonal necrosis of the posterior wall of the left ventricle.

In two cases of suspected myocardial infarction of estimated clinical age one to five hours, the macroenzymatic examination with NBT did not reveal any myocardial damage. Necropsy examination of these two hearts showed fresh thrombotic occlusion of the left anterior descending coronary artery, and moderate atheroma of the other two major coronary arteries. There was no naked eye evidence of myocardial infarction and microscopic examination showed apparently normal myocardial fibres (Grade A).

C. Suspected myocardial infarction of estimated clinical age five to twelve hours

Out of the twenty-one hearts examined by the NBT test in this clinical age group of five to twelve hours, the macroenzymatic reaction was negative (positive result showing damaged heart muscle) in twenty hearts (Table 20; Figures 34, 35, 36, 37). In thirteen of these twenty hearts, conventional autopsy did not show apparent gross changes characteristic of myocardial damage. Microscopic examination of the mirror-image surface of the lesion showed no histologic evidence of recent myocardial damage (Grade A) in three hearts, while the pre-inflammatory signs (Grade B) were seen in seven hearts. Neutrophil polymorph infiltration (Grade C) was demonstrated in three hearts.

Naked eye evidence of recent myocardial infarction was seen in five out of the twenty NBT-positive hearts, and clear necrosis of heart muscle in two hearts. In these seven hearts, the gross enzyme histo-

Table 20. Nitroblue tetrazolium staining of suspected myocardial infarcts with estimated clinical age five to twelve hours

NBT-Macro-reaction	Number of cases (Total 21)	Naked eye infarct	Microscopic grade				Coronary artery thrombosis
			A	B	C	D	
			Number of cases				
Transmural	11	5	1	5(1)	3(1)	2(2)	9
Zonal	4	1	2	0	2(1)	0	3
Laminar	1	0	0	1	0	0	1
Papillary Muscle	2	0		2	0	0	1
Mixed	2	1	0	1(1)	1(1)	0	1
Total	20	7	3	9(2)	6(3)	2(2)	15
Negative	1	0	1				1

The number between brackets represents the number of cases with naked eye infarction at autopsy

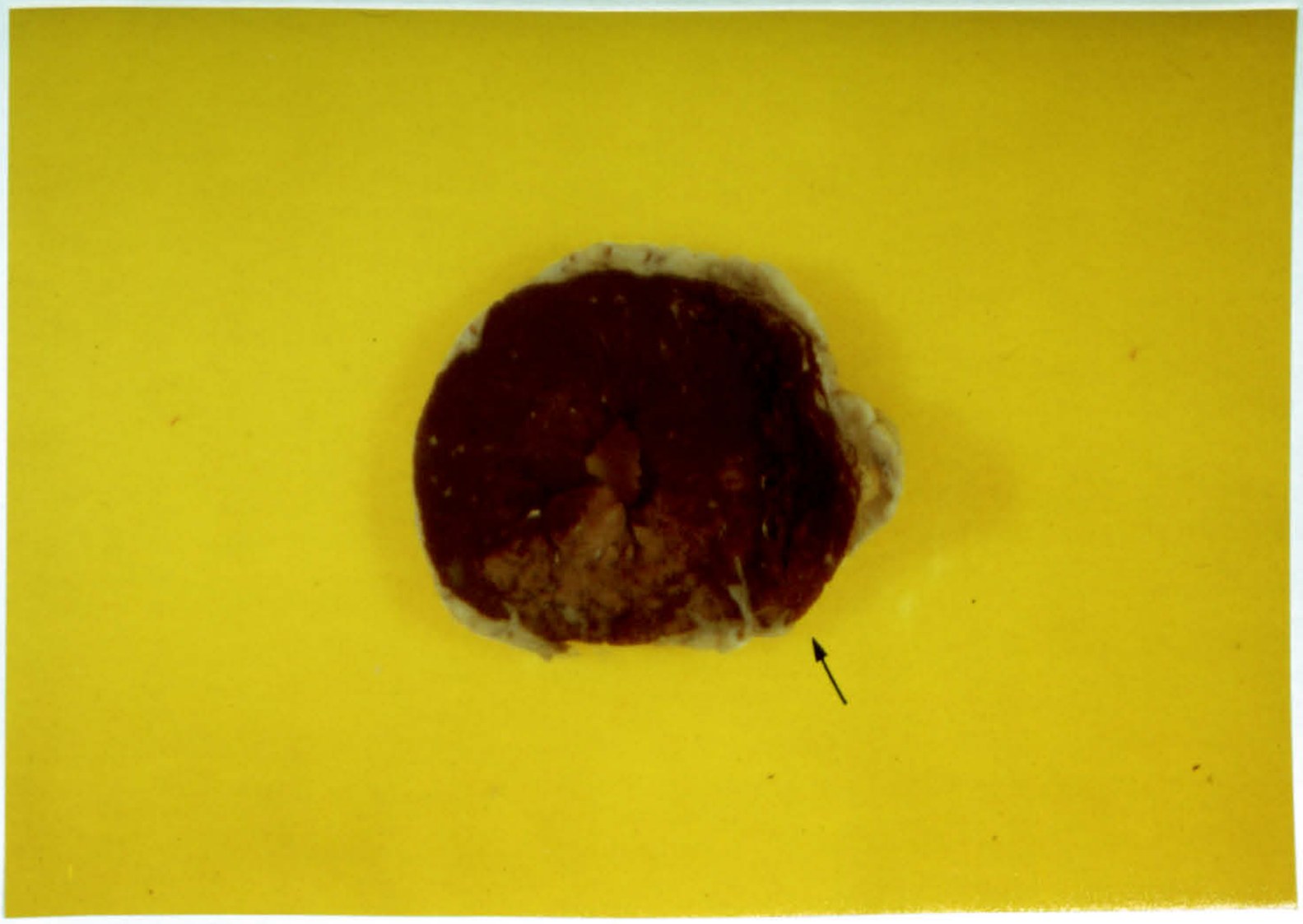


Figure 34: NADPH tetrazolium reductase macrostaining of a heart slice from a woman of 67 years with a 5½ hour clinical history. Recent postero-septal transmural infarct of the left ventricle and right ventricle (arrow), and of the posterior papillary muscle. Occlusion of the right coronary artery by a recent thrombus.

Figure 35: NADH tetrazolium reductase macrostaining of a heart slice from a man of 44 years with a clinical history of 6 hours. Recent postero-septal transmural infarct of the left ventricle and right ventricle (arrow). Occlusion of the right coronary artery by a recent thrombus.

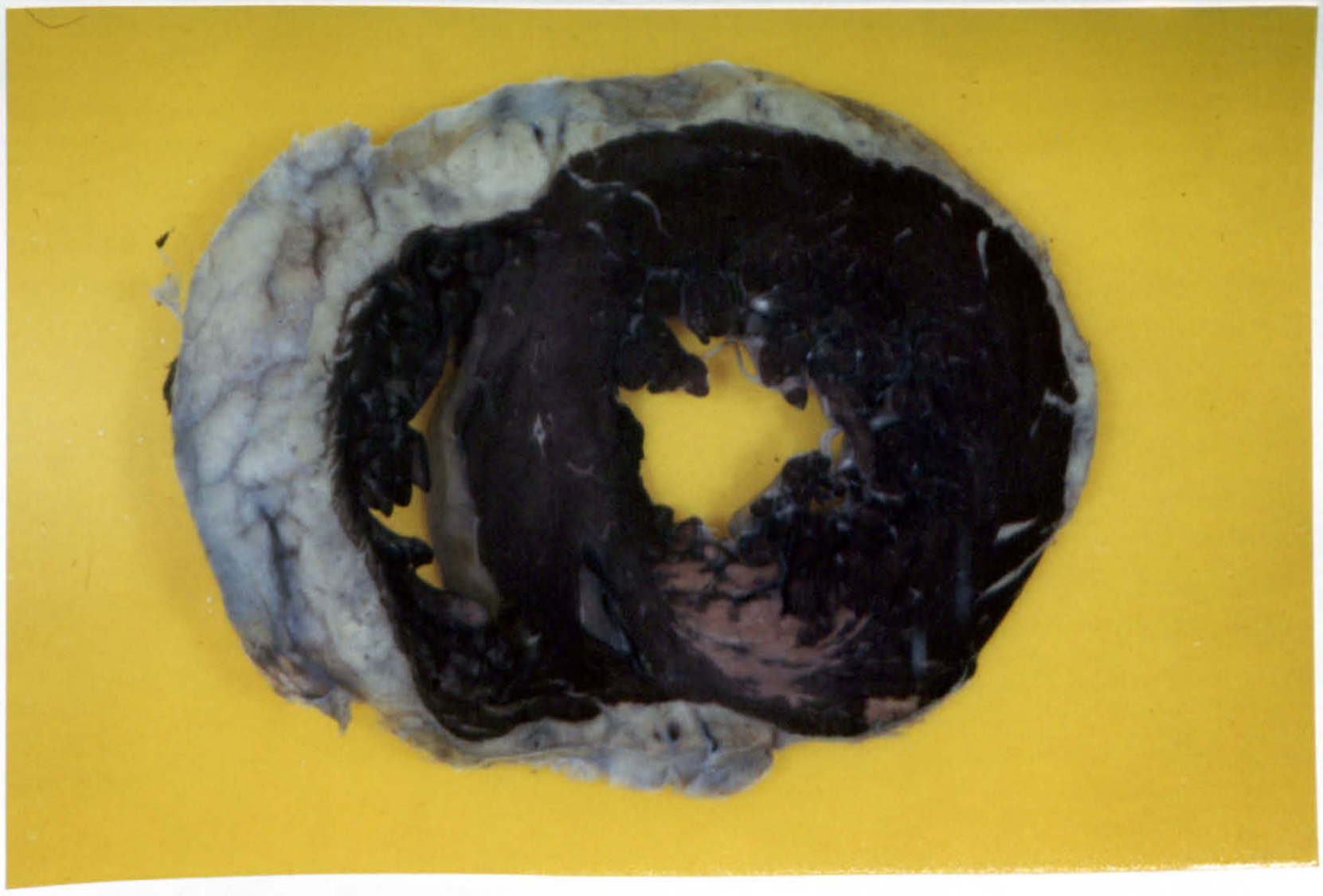


Figure 36: Malate dehydrogenase macrostaining of a heart slice from a man of 55 years with a clinical history of 9 hours. Recent transmural infarct of the posterior wall of the left ventricle. Occlusion of the right coronary artery by a recent thrombus.

Figure 37: Non-specific dehydrogenase macrostaining of a heart slice from a woman of 61 years with a clinical history of 11 hours. Recent postero-lateral zonal infarct of the left ventricle. Occlusion of the left circumflex coronary artery by a recent thrombus.

chemistry revealed the real extent of the myocardial damage by outlining the boundaries of the infarct. One heart was of a fifty-eight year old male patient who was transferred to the intensive care unit for monitoring of an acute myocardial infarction. He died six hours later. Autopsy performed forty-eight hours after death showed a yellowish infarct of the postero-lateral wall of the left ventricle. Macroscopic enzyme staining of the heart revealed a superimposed recent laminar extension of the myocardial damage (figure 38), which was not identified clinically and was not suspected or recognised at autopsy, and was of Grade A histology.

Examination of the coronary arteries in the hearts included in this clinical age group of five to twelve hours thrombotic occlusion in sixteen out of twenty-one hearts. In one of these hearts, the NBT test was negative. In four hearts the coronary arteries showed advanced atheromatous changes. One last case of a sixty-six year old male deserves a particular comment. He was admitted to the hospital twenty-four hours before death in respiratory failure, which was confirmed by analysis of blood gases. Twelve hours before death, an intravenous drip infusion was set up which was disconnected as the patient was disorientated and trying to get out of bed, and then another drip was set up. Examination of the coronary arteries at necropsy, which was performed thirty-six hours after death, showed moderate atheroma of the left and right coronary arteries. A froth of blood and air escaped on dissecting the vessels. Likewise beads of air embolism were observed over the cerebral hemispheres. There was a small area of equivocal pallor of the anterior wall of the left ventricle. The macroenzymatic

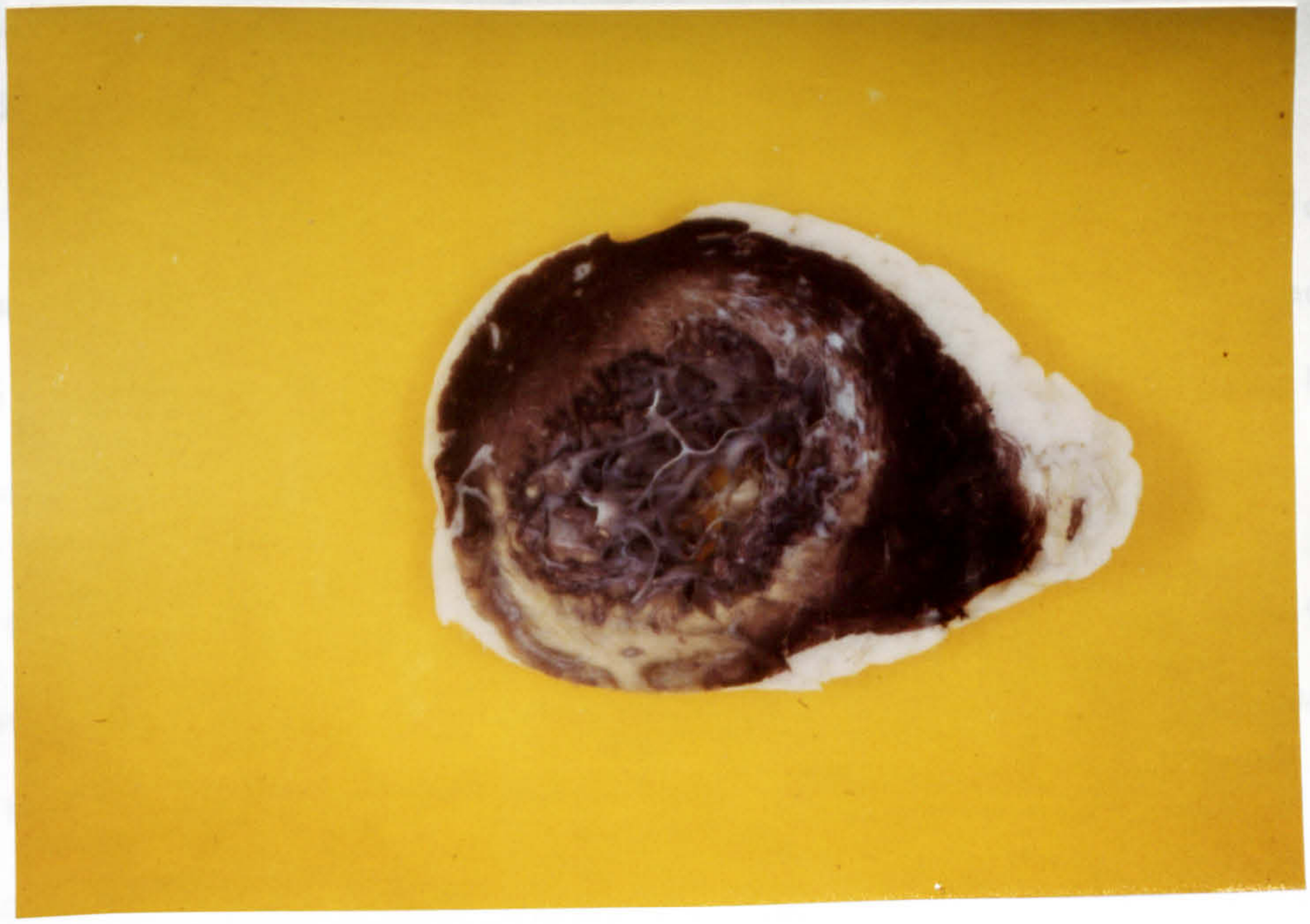


Figure 38: Non-specific dehydrogenase macrostaining of a heart slice from a man of 58 years admitted to the intensive care unit for monitoring of myocardial infarction. The yellow infarct of the postero-lateral wall of the left ventricle was recognised at autopsy. Recent circumferential extension of the myocardial damage, unrecognised at autopsy. Note the patchy fibrosis of the antero-septal wall. Occlusion of the left anterior descending and right coronary arteries by recent thromboses.

reaction in this heart showed a clearly positive NBT test (Figure 39), and histologically the damaged area was of Grade B.

D, <u>Suspected myocardial infarction of estimated clinical age twelve to twenty-four hours</u>

The hearts from eleven cases of recent myocardial infarction of estimated clinical age twelve to twenty-four hours were included in this group, and were subjected to the macroenzymatic reaction. The NBT test was positive in all hearts (Table 21; Figures 40, 41).

Out of these eleven hearts, naked eye evidence of the lesion at autopsy was absent in six hearts, but was recognised in the remaining five hearts, in which the boundaries of the damaged area of the heart were not demarcated except after the macroenzymatic reaction was performed. Histologically, all eleven hearts showed evidence of myocardial damage.

Fresh thrombotic occlusion of the coronary arteries were found in seven hearts, and occlusive atheroma in three hearts. One further heart was obtained from a fifty-three year old male patient who died fifteen hours after surgical replacement of a ruptured ascending aortic aneurysm and aortic valve replacement. The ostia of the two main coronary arteries were compressed by blood which was leaking at the site of operation and had tracked along the wall of the coronary arteries, which otherwise showed minimal to moderate atheromatous changes. In this heart there was no naked eye evidence or suspicion of myocardial damage. Gross enzyme histochemistry revealed a weak negative enzymatic reaction of the postero-lateral wall of the left ventricle and of the posterior septum as well as generalized ischaemia of the papillary muscles

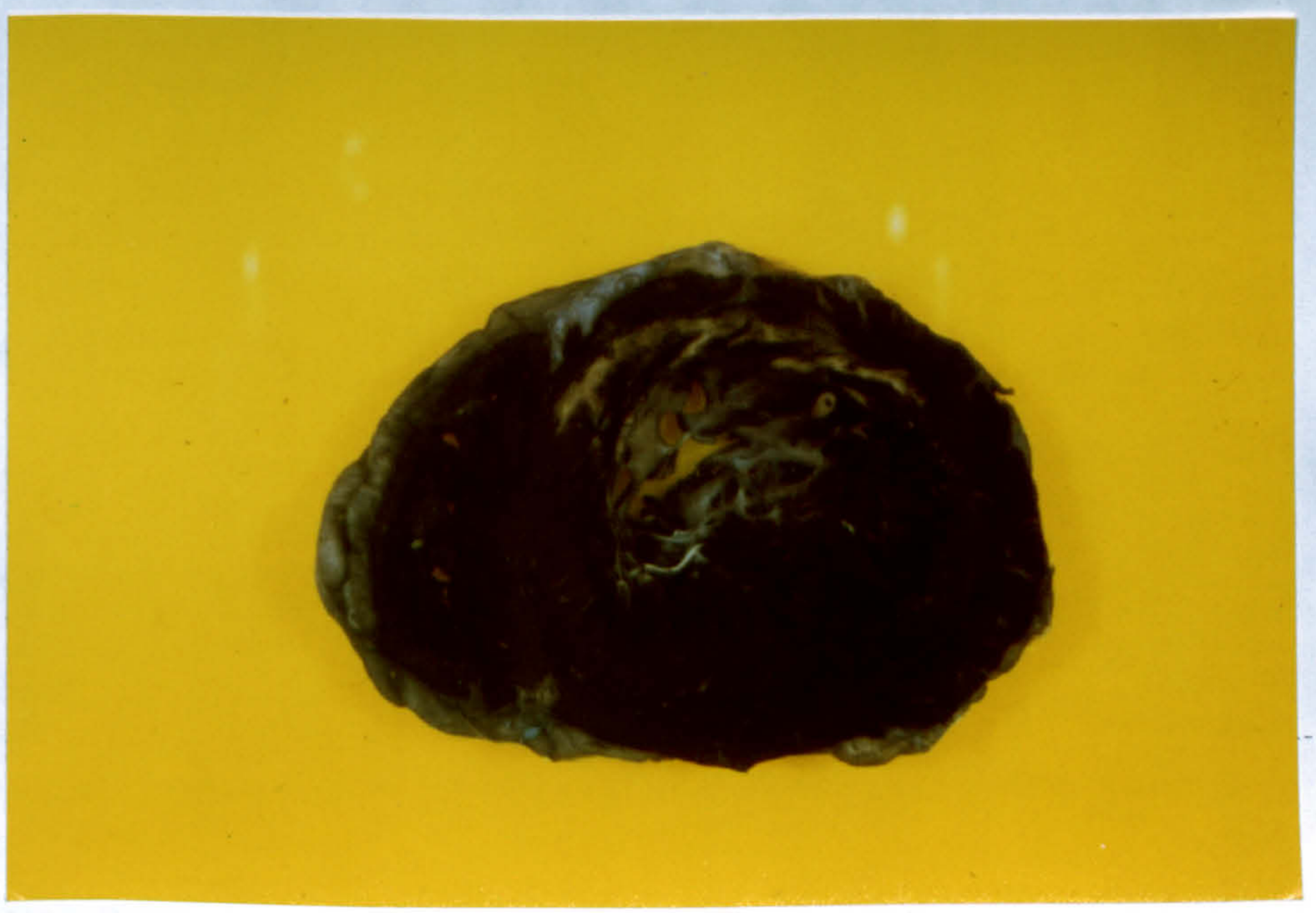


Figure 39: Non-specific dehydrogenase macroreaction in a heart slice from a man of 66 years. Coronary air embolism had complicated the course of transfusion 12 hours before death. Recent antero-septal zonal infarct of the left ventricle.

Table 21. Nitroblue tetrazolium staining of myocardial infarcts of estimated clinical age twelve to twenty-four hours

NBT Macro-reaction	Number of cases (Total 11)	Naked eye infarct	Microscopic grade				Coronary artery thrombosis
			A	B	C	D	
			Number of cases				
Transmural	6	3	0	1	3(1)	2(2)	4
Zonal	4	2	0	1	2(1)	1(1)	2
Laminar	0						
Papillary muscle	0						
Mixed	1	0	0			1	1
Total	11	5	0	2	5(2)	4(3)	7
Negative	0						

The number between brackets shows the number of cases with naked eye infarct at autopsy.

Figure 40: Non-specific dehydrogenase macroreaction in a heart slice from a man of 74 years with a clinical history of 14 hours. Thrombotic occlusion of the right coronary artery and occlusive atheroma (arrow) of the left anterior descending coronary artery. Recent circumferential infarct of the left ventricle. Coarse fibrosis of the posterior ventricular wall.

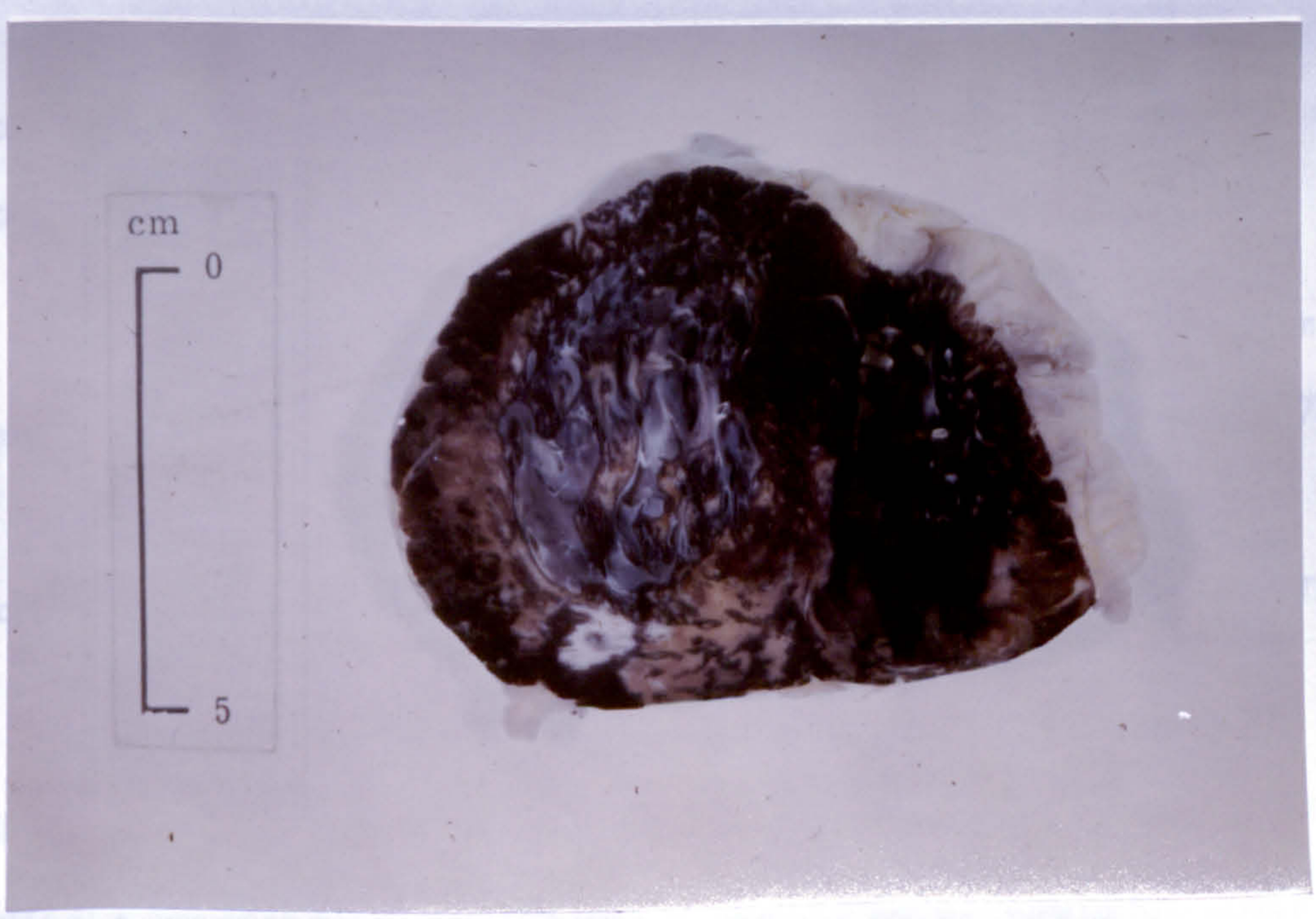


Figure 41: Heart slice from a woman of 70 years with a clinical history of 18 hours. Occlusion of the right coronary artery by a recent thrombus. Recent postero-lateral transmural infarct of the left ventricular wall, of the posterior septum and of the posterior wall of the right ventricle revealed by the non-specific dehydrogenase macroreaction. A fibrous patch is seen in the posterior wall of the left ventricle.

(Figure 42). Histological examination showed the lesion to be of Grade A.

The macroenzymatic study of all suspected cases of recent myocardial infarction

The overall results obtained in this study on the application of gross enzyme histochemistry for the macroscopic identification of early myocardial infarcts are summarized in Table 22.

The macroenzymatic reaction using the nitroblue tetrazolium test revealed areas of recent myocardial damage in eighty-four (77.7%) out of the one hundred and eight hearts suspected of harbouring a myocardial infarct of estimated clinical age under 24 hours duration.

In sixty-four (76.1%) out of the eighty-four NBT-positive hearts, naked eye evidence of the lesion was not recognised or suspected at ordinary macroscopic evaluation. Twenty-three cases of these were of estimated clinical age under one hour; twenty-two hearts of one to five hours; thirteen hearts of five to twelve hours, and six hearts of twelve to twenty-four hours. Histologic evidence of the lesion was absent in thirty-four hearts. These included seventeen cases of clinical age under one hour, fourteen cases of one to five hours, and three cases of five to twelve hours. All cases of twelve to twenty-four hours showed histologic changes of myocardial infarction.

The macroscopic nitroblue tetrazolium test was negative in twenty-four hearts (22.2%). None of these hearts showed gross or histologic evidence of a lesion.

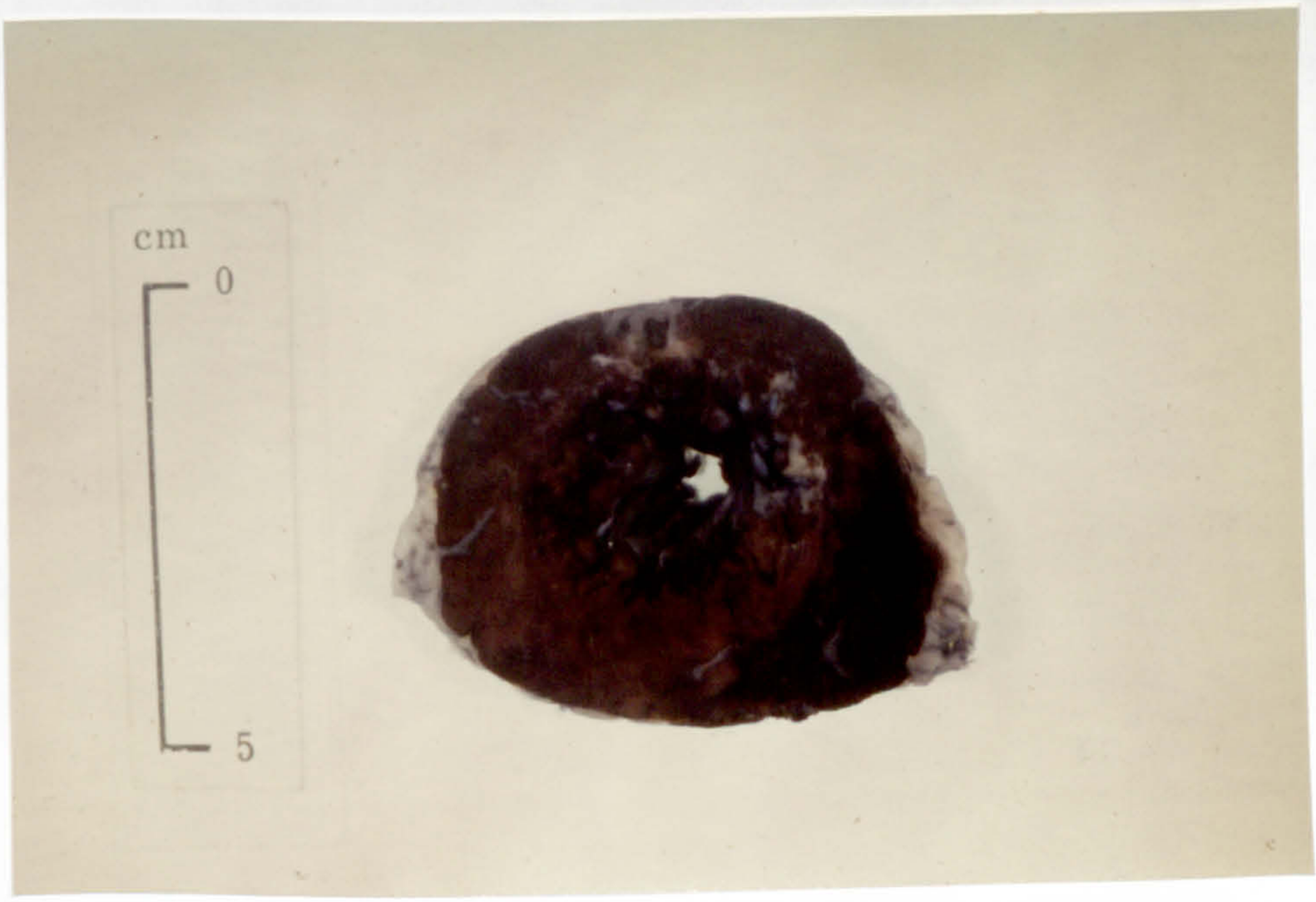


Figure 42: Non-specific dehydrogenase macrostaining of a heart slice from a 53-year old man. The ostia of the main left and right coronary arteries were compressed by blood leaking from site of operation, complicating surgical replacement of ruptured ascending aortic aneurysm and aortic valve replacement. A transmural negative enzymatic reaction of the postero-lateral wall of the left ventricle and of the posterior septum, including the papillary muscles. The right ventricle is darkly stained.

Table 22. Nitroblue tetrazolium gross examination of the 108 suspected cases of recent myocardial infarction in which an occlusion was found

Clinical age	Total Number	NBT test		Naked eye infarction		Microscopic grade		Coronary artery thrombosis	
		positive (+ve)	negative (-ve)	NBT +ve	NBT -ve	NBT +ve	NBT -ve	NBT +ve	NBT -ve
Under one hour	48	27	21	4/27	2/21	17A, 8B 2C, 0D	21A	22/27	19/21
1 - 5 hours	28	26	2	4/26	0/2	14A, 9B 2C, 1D	2A	19/26	2/2
5 -12 hours	21	20	1	7/20	0/1	3A, 9B 6C, 2D	1A	15/20	1/1
12-24 hours	11	11	0	5/11	0/0	0A, 2B 5C, 4D	0	7/11	0
Total	108	84	24	20/84	2/25	34A,28B 15C, 7D	24A	63/84	22/24

III. MISCELLANEOUS GROUP OF MYOCARDIAL NECROSIS

The macroenzymatic histochemical reaction using the nitroblue tetrazolium test was applied to ten hearts which were classified as miscellaneous because they were obtained from patients who died of illness other than myocardial infarction, but in whom it was thought that hypoxia and shock might have caused damage to the myocardium. In none of these cases was acute myocardial infarction diagnosed or suspected clinically. At autopsy, the coronary arteries were not significantly diseased and none of them showed occlusive thrombosis.

Case Number 1: Severe megaloblastic anaemia

A fifty-year old female patient suffered from viral encephalitis nine years ago and this led to postencephalitic dementia. She had also suffered from megaloblastic anaemia of dietary origin for four years, for which she was admitted to the hospital several times for treatment with folic acid and vitamin B_{12}. The last admission was two days before death. Haematological investigation showed severe anaemia; packed cell volume 6%; haemoglobin 2 g. Four units of packed cells were transfused, but the patient developed pyrexia, a rapid pulse and a high blood pressure. She died suddenly.

Necropsy was carried out twenty-four hours after death. The heart weight was 320g. The myocardium showed haemorrhagic spots in the posterior wall of the left ventricle. Macroenzymatic examination revealed an area of necrosis in the posterior wall of the left ventricle (Figure 43) which was of Grade B histology. The diminished oxygen-carrying power of the blood appears to be responsible for the myocardial damage.

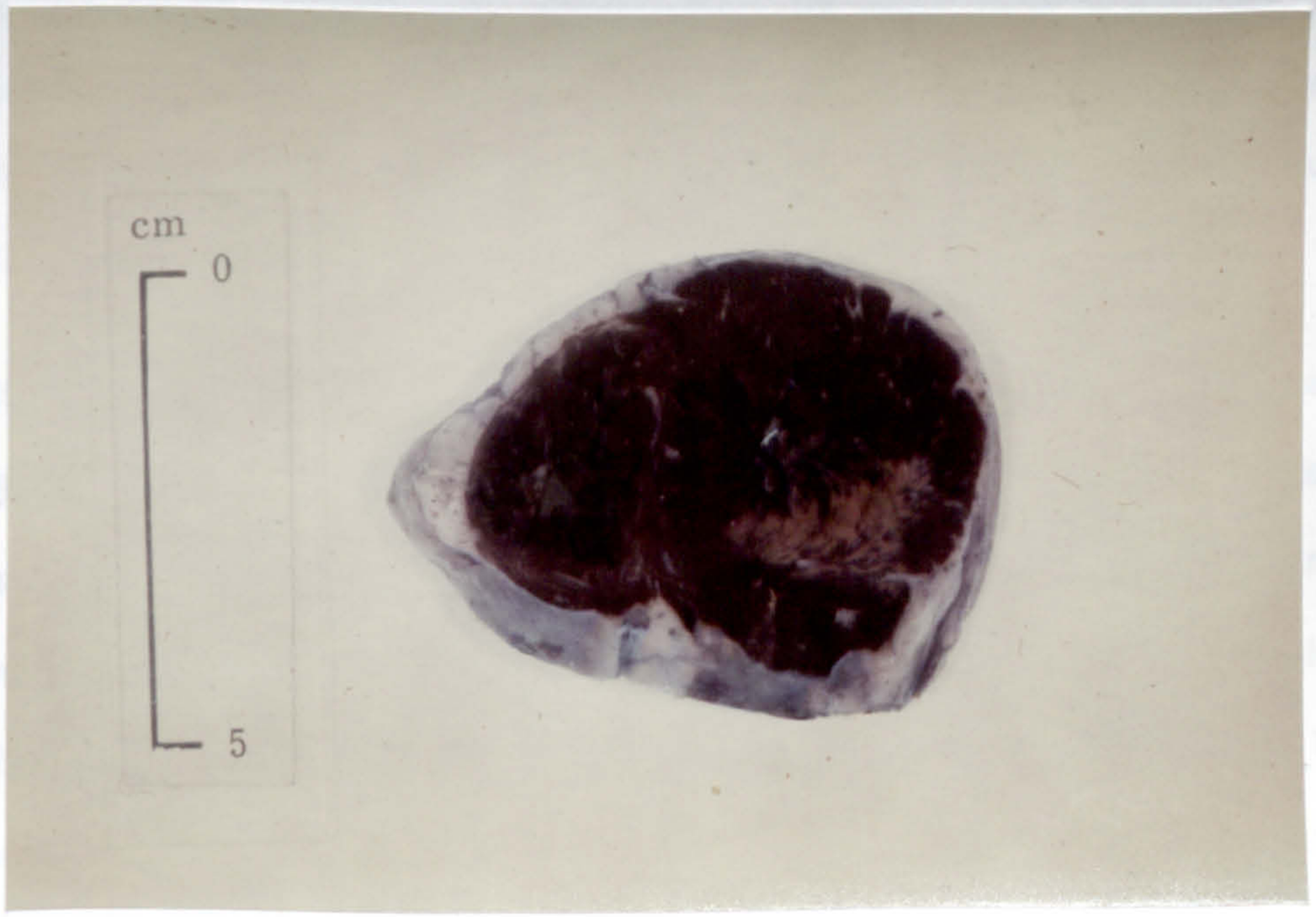


Figure 43: A heart slice from a 50-year old woman showing near transmural necrosis of the posterior wall of the left ventricle as revealed by non-specific dehydrogenase macroreaction. A case of severe megaloblastic anaemia.

Case Number 2: Congestive Cardiomyopathy

An eighteen year old male patient presented at the hospital following an episode of syncope. He had an abnormal ECG, and had some signs of right heart failure. A chest X-ray showed an enlarged heart. His condition was controlled medically with digitalis and diuretics. He was discharged to be readmitted after eighteen days with supraventricular tachycardia which converted to atrial fibrillation. He had a cardiac arrest two days before death, from which he was successfully resuscitated, but died following a further cardiac arrest.

Autopsy was performed thirty-six hours after death. The heart weight was 490g. The right and left ventricles were both dilated and coarse fibrosis was visible in the wall of the left ventricle. Macroenzymatic examination showed extensive patchy fibrosis of the left and right ventricles. Fibrosis of the latter was overlooked at autopsy. The interventricular septum was relatively less involved by the patchy fibrosis (Figure 44). Electrocardiographic changes consistent with infarction have been reported in the literature in congestive cardiomyopathy. The extensive fibrosis seen in this case might well represent healed myocardial infarction.

Case Number 3: Rheumatic mitral stenosis

A seventy-three year old male patient, who had had bronchitis for many years, was admitted to the hospital with bronchopneumonia. He died unexpectedly two days later.

Autopsy was performed forty-eight hours after death. The heart weight was 375g. There was advanced mitral stenosis following an old

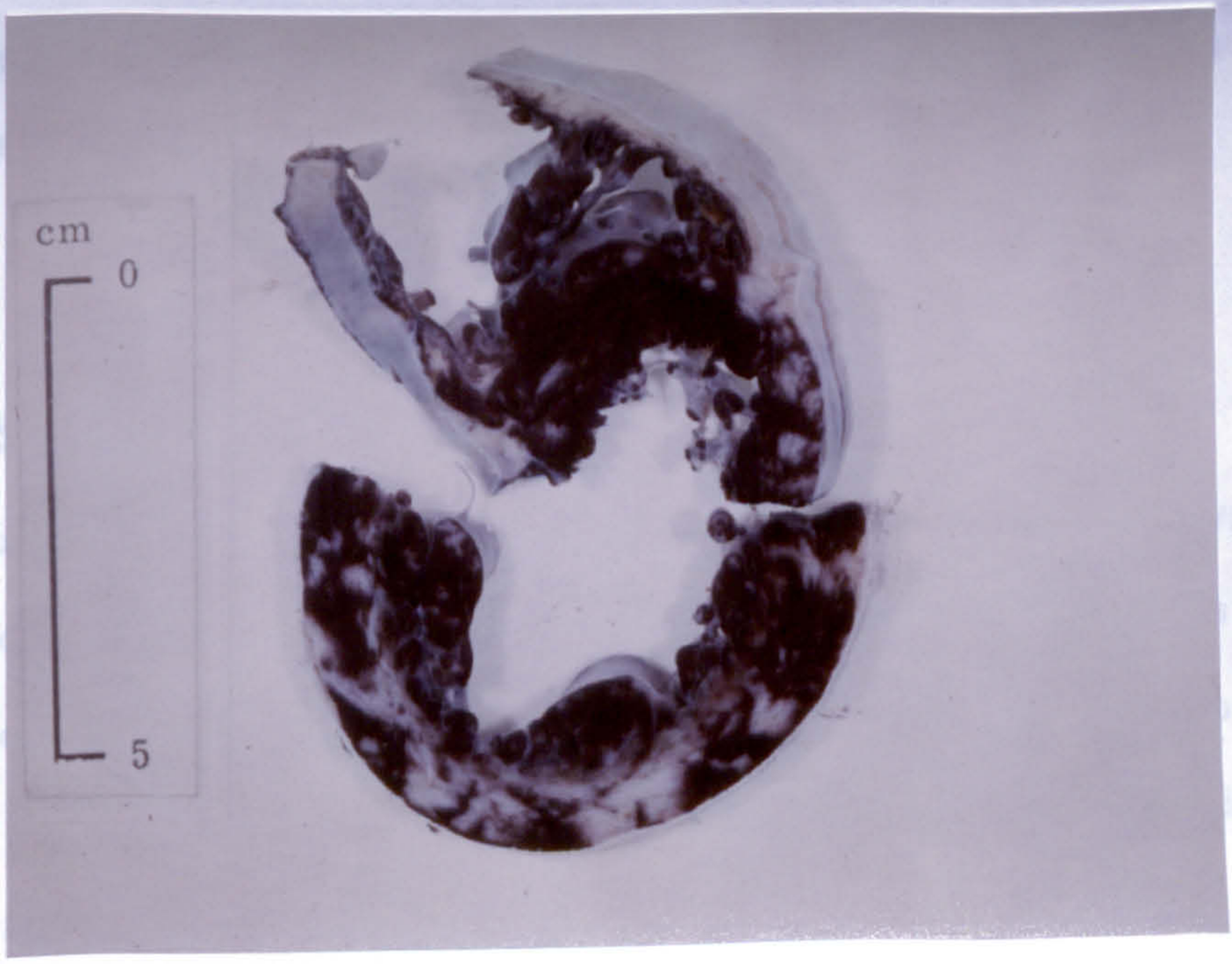


Figure 44: A heart slice stained with B-hydroxybutyrate dehydrogenase from a man of 18 years. Extensive fibrosis of the left ventricle seen at autopsy and well outlined by the enzymatic macroreaction. Fibrosis of the right ventricle was overlooked at autopsy. A case of congestive cardiomyopathy.

rheumatic endocarditis, and early stenosis of the aortic valve. The myocardium was apparently normal. Macroenzymatic examination revealed a laminar necrosis of the myocardium of the left ventricular wall (Figure 45), which was of Grade B histology. In this case, perhaps the associated increased pulmonary vascular resistance might have resulted in low cardiac output which, together with the associated aortic stenosis, could have led to poor coronary perfusion.

Case Number 4: Calcific aortic stenosis.

A sixty-eight year old male patient, with a history of dyspnoea on effort and paroxysmal nocturnal dyspnoea, recently suffered fainting attacks, especially after effort, for which he was admitted to the hospital. His condition improved with treatment, but he died unexpectedly one week after admission.

Autopsy was performed forty-eight hours after death. The heart weight was 440g. The myocardium showed left ventricular hypertrophy. The aortic valve was heavily calcified and congenitally bicuspid. NBT test revealed zonal necrosis of the left ventricular wall (Figure 46), which was unrecognised at autopsy, and was of Grade B histology. In this case it seems that coronary blood flow was insufficient for the increased left ventricular work.

Case Number 5: Carcinoma of the stomach

A sixty-four year old female presented with a history of extreme weakness, loss of appetite and loss of weight. Barium meal showed

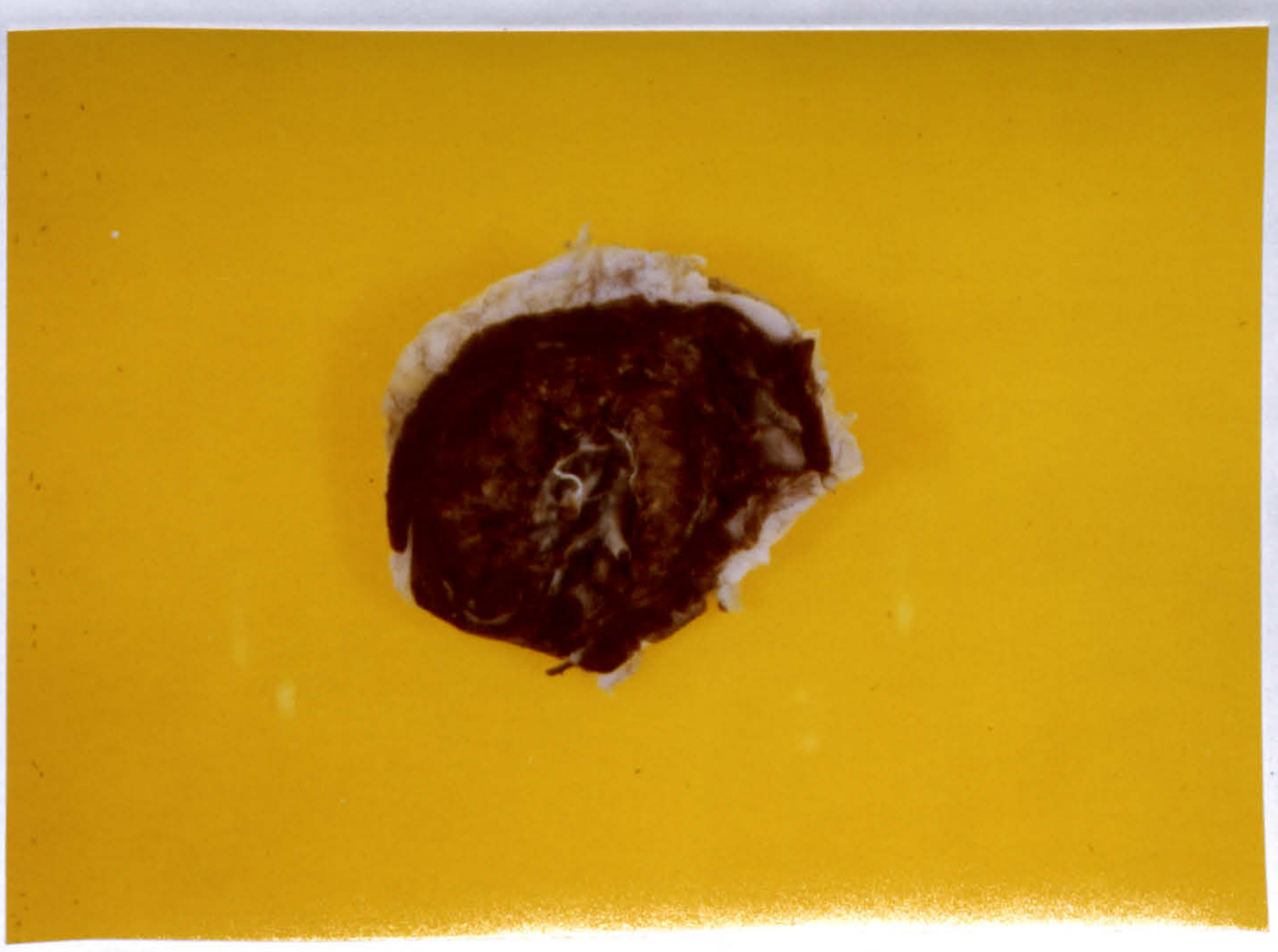


Figure 45: Laminar necrosis of the left ventricular wall as revealed by the non-specific dehydrogenase macro-reaction. Heart slice from a man of 73 years with rheumatic mitral stenosis.

Figure 46: Two opposing heart slices from a man of 68 years. Necrosis of the left ventricular wall (zonal) and right ventricular papillary muscles, revealed by succinate dehydrogenase (left slice) and non-specific dehydrogenase (right slice) macroreaction. A case of calcific aortic stenosis.

narrowing and deformity of the pyloric antrum. She died two weeks after admission.

Autopsy was performed thirty-six hours after death. The heart weight was 383g. A pale small area was seen in the posterior wall of the left ventricle, which showed a positive NBT test (Figure 47), and histologically was of Grade B. There was an annular scirrhous carcinoma of the pyloric antrum and pyloric canal. Possibly a severe anaemia and shock complicating carcinoma of the stomach in its terminal stage might well be the underlying cause.

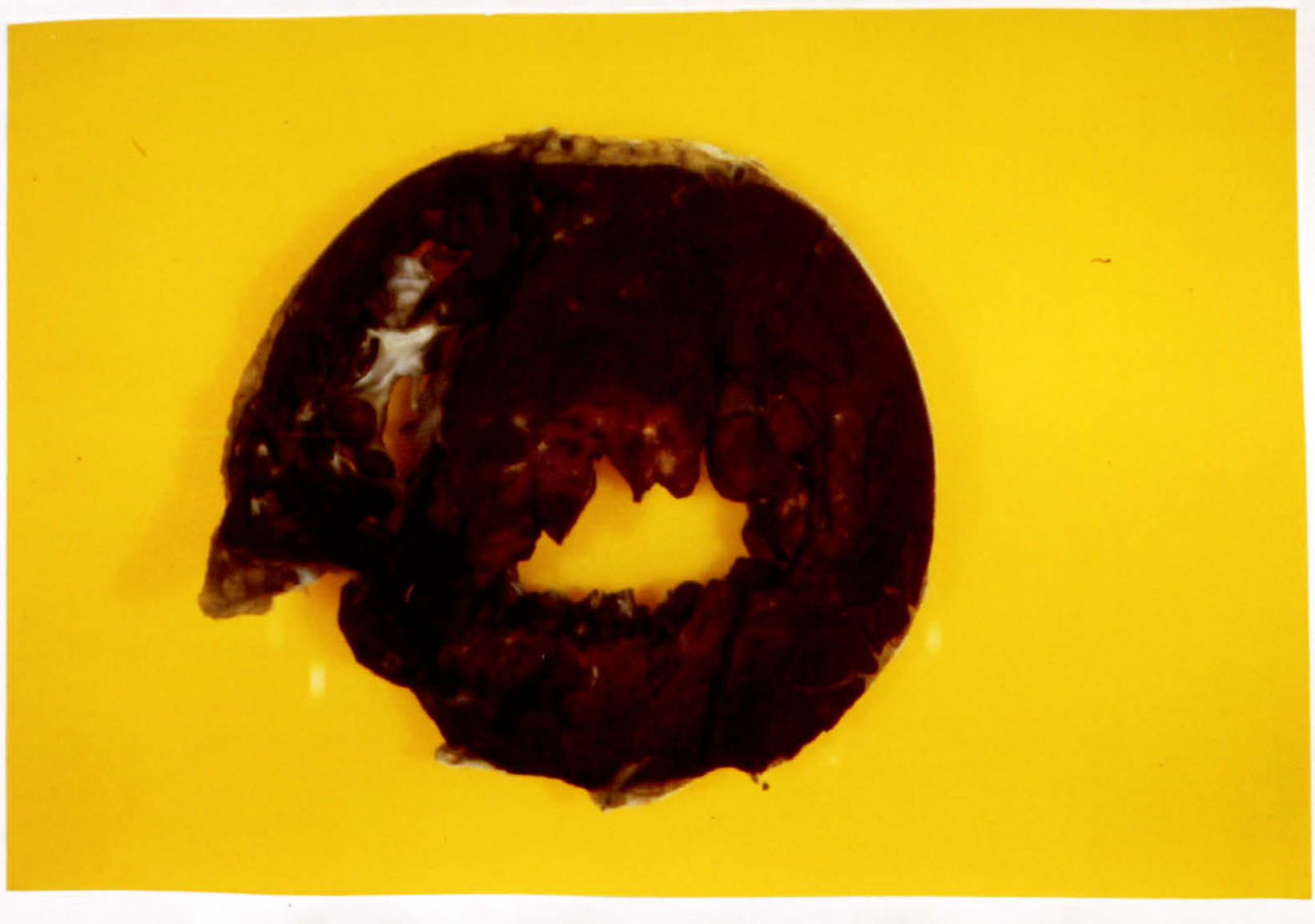
Case Number 6: Subarachnoid haemorrhage

This forty-two year old female was admitted to the hospital one day before death because of sudden collapse after severe pain in the back of the head and neck. The pupils were fixed and dilated, tendon reflexes were absent and there was no response to painful stimuli. Lumbar puncture revealed gross bleeding. She suffered cardiac arrest one hour after admission, which returned to sinus rhythm, arrested again two hours later and died.

Necropsy was performed seventy-two hours after death. The heart weight was 345g. The NBT test revealed a laminar negative macroenzymatic reaction of the left ventricle and of the papillary muscles (Figure 48). There was no naked eye evidence of the lesion, and histologically it was of Grade A. There was subarachnoid and interventricular haemorrhage from a ruptured aneurysm of the anterior communicating artery. Possibly myocardial necrosis was caused by hypotensive shock. This is discussed further in Chapter V.

Figure 47: Two opposing heart slices from a woman of 64 years showing an area of necrosis of part of the left ventricular wall, revealed by non-specific dehydrogenase (added coenzyme II NADP; left slice) and iso-citrate dehydrogenase (right slice) macroreaction. A case of carcinoma of the stomach.

A



B

Figure 48: Heart slices from a woman of 52 years. Slice A shows subendocardial zonal necrosis of the septum and the left ventricular papillary muscles, revealed by β-hydroxybutyrate dehydrogenase macrostaining. Slice B shows laminar necrosis of the left ventricle and of the papillary muscles of both ventricles, revealed by non-specific dehydrogenase macroreaction. Many residual foci with intact enzyme reaction are seen within the lesion. A case of subarachnoid haemorrhage.

Case Number 7: Coronary artery hypoplasia

A sixty-six year old man was admitted with supraventricular tachycardia, severe left ventricular failure and unrecordable blood pressure. He was given D.C. shock four times; he arrested and was resuscitated. His condition remained unstable, and he died six weeks after admission. ECG recorded before death showed ST-segment and T wave changes.

Autopsy was performed thirty-two hours after death. The heart weight was 570g. The heart was flaccid and markedly dilated. A diminution in NBT staining of the interventricular septum, of the posterior wall of the left ventricle at the junction with the posterior septum, and of the posterior right ventricular wall was observed (Figure 49). The lesion was of Grade B histology. The coronary arteries were not diseased but were of small calibre which may eventually have led to inadequate poor perfusion.

Case Number 8: Acute corrosive poisoning

A fifty-year old female had suffered from depression for the last five years. She was found in the bathroom with her wrists slashed, and apparently had swallowed a large dose of a corrosive substance.

Autopsy was performed forty-eight hours after death. The heart weight was 350g. There was no myocardial damage apparent to the naked eye. The NBT test revealed a small area of myocardial damage in the lateral wall of the left ventricle (Figure 50), which histologically was of Grade A. The stomach and oesophagus were stained intensely blue and

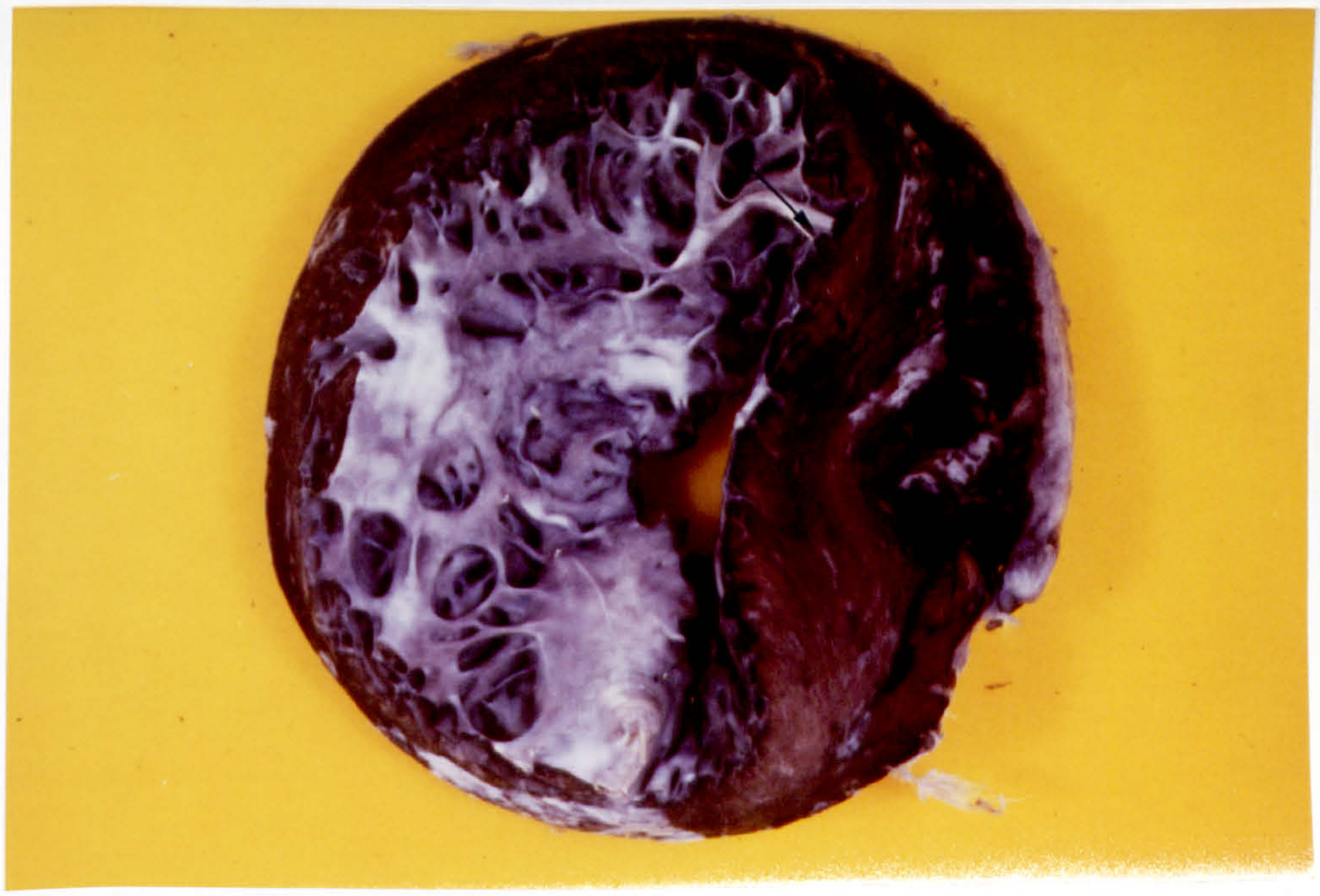


Figure 49: Heart slice stained with NADPH tetrazolium reductase from a man of 66 years. Full thickness diminution in staining intensity of the posterior septum (bottom) and, subendocardially, of the anterior septum (arrow). The posterior wall of the left ventricle is necrotic at its junction with the posterior septum, and is the posterior wall of the right ventricle. A case of coronary artery hypoplasia.

Figure 50: Two opposing heart slices from a woman of 50 years. A small area of myocardial necrosis of the lateral wall of the left ventricle, revealed by glucose-6-phosphate dehydrogenase (left slice), and non-specific dehydrogenase (with added coenzyme II NADP, right slice) macroreaction. .The latter also shows involvement of the papillary muscles. A case of acute corrosive poisoning.

showed corrosive changes. The stomach wall and the left diaphragm had been fixed by corrosive action. It seems that the condition was complicated by shock and hypotension, again leading to poor coronary perfusion.

Case Number 9: Chest infection

A seventy-seven year old female patient, who had had bronchitis for many years, was admitted with pneumonia and severe respiratory distress. Her condition gradually deteriorated and, about six hours before death, her pulse became inpalpable and her blood pressure unrecordable.

Autopsy was performed twenty-seven hours after death. The heart weight was 400g. Macroenzymatic examination revealed a zonal negative reaction in the left and right ventricular walls (Figure 51), which was not recognised at autopsy and which was of Grade A histology. Both lungs were congested and emphysematous. The hypotension and the unrecordable blood pressure six hours before death seem to have caused poor coronary perfusion.

Case Number 10: Ellis-Van Creveld Syndrome

A forty-nine year old male patient, diagnosed as Ellis-Van Creveld syndrome with polydactly, shortening of the long bones of the arms and legs, and a heart defect. He was admitted three days before death with a history of chest pain for three weeks, and increasing dyspnoea

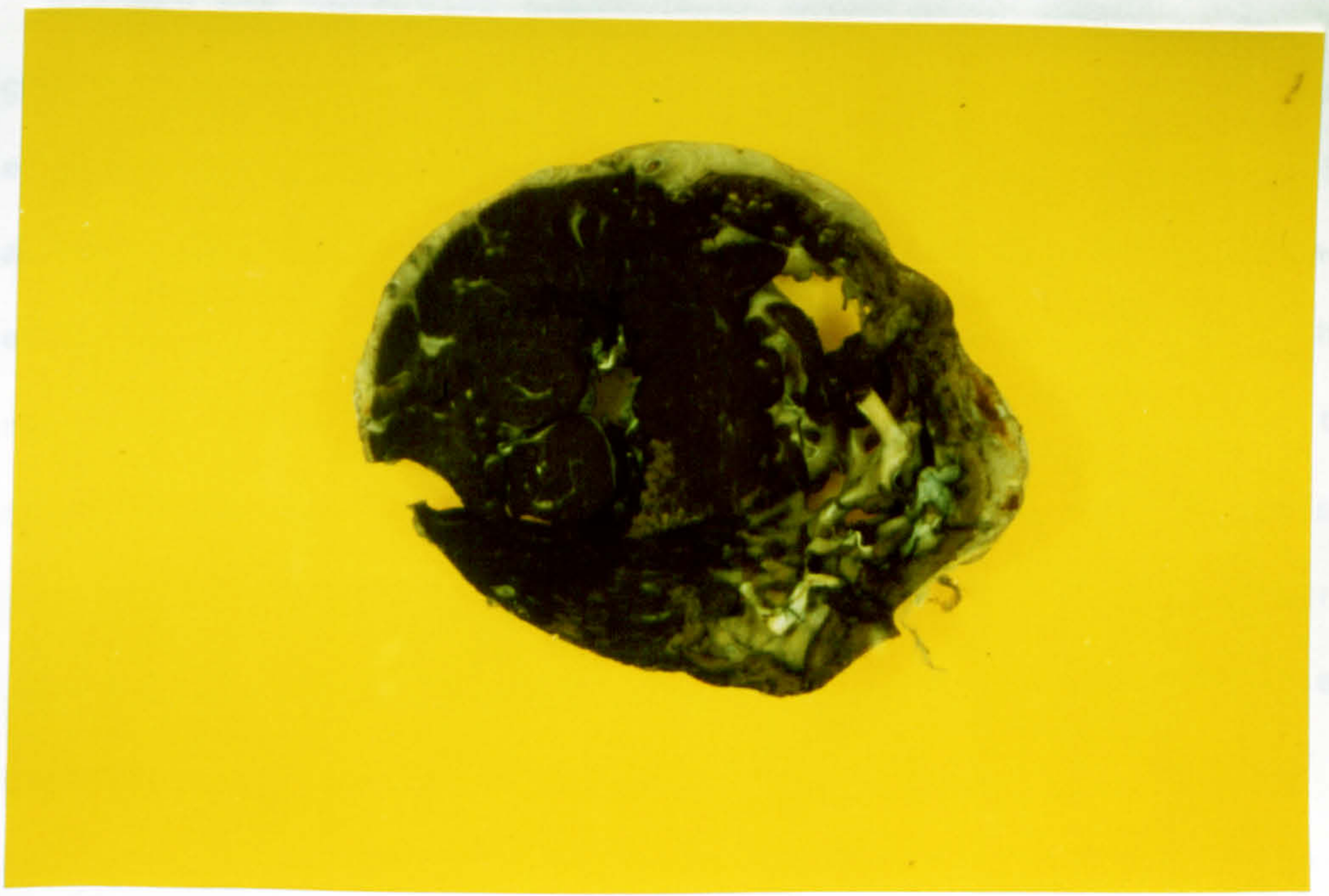


Figure 51: Heart slice from a woman of 77 years. Zonal necrosis of the posterior septum and necrosis of almost all the right ventricular wall, revealed by the NADH tetrazolium reductase macroreaction. A case of chest infection.

since with haemoptysis. He presented with signs of congestive heart failure and a chest infection. His condition slightly improved on treatment, but he died three days after admission.

Autopsy was performed twenty-four hours after death. The heart weight was 550g; the heart was enlarged. There was a single atrium (i.e. gross atrial septal defect), and the left ventricle was slightly dilated. The right ventricle was enlarged, and dilated and showed hypertrophy of its wall, in evidence presumably of a left to right shunt. Macroenzymatic examination revealed recent myocardial damage of the postero-lateral wall of the left ventricle and of the posterior papillary muscles, and diffuse fibrosis of the right ventricular wall(Figure 52). The recent myocardial damage of the posterolateral wall of the left ventricle was not recognised or suspected at autopsy, and was of Grade A histology. As a result of the left to right shunt, the pressure in the right side of the heart may have increased until the shunt reversed. The resulting cardiac failure presumably caused an overall fall in coronary perfusion.

Figure 52: Heart slice from a man of 49 years. The non-specific dehydrogenase macroreaction revealed recent postero-lateral zonal necrosis of the left ventricle including the papillary muscles. Diffuse fibrosis of the right ventricular wall. A case of Ellis-Van Creveld syndrome.

IV. THE EVALUATION OF THE CONDITIONS THAT PROVIDE THE MAXIMUM NBT DEHYDROGENASE MACROREACTION AS APPLIED IN THIS STUDY

A. The role of coenzyme NAD and the respiratory chain inhibitor, cyanide, in the histoenzymatic macroreaction

Of the various NBT incubating media for dehydrogenase macroreactions (Table 14, page 90), the non-specific dehydrogenase incubating medium using the heart's own endogenous substrate with added NAD (nicotinamide adenine dinucleotide; coenzyme I) was observed to give optimal results for the gross detection of myocardial infarction. The inclusion of the respiratory chain inhibitor, cyanide, in the incubating medium to direct electron transfer away from the cytochrome oxidase system towards the tetrazolium salt has increased the final colour product and increased the consistency of the results. This redirection of electron transport results from the poisoning of cytochrome oxidase by cyanide. This use of NAD and cyanide is a new modification introduced in the present study.

To prove the effective role of NAD and of cyanide in the NBT enzymatic macroreaction, normal hearts obtained from subjects who died in road traffic accidents were tested. The death-necropsy interval was five to forty-eight hours. Transversely cut heart slices and pieces of heart tissue from the same heart were incubated in various NBT incubating media, using the same stock incubating solution and under identical conditions of incubation (temperature, time and the same shaking of containers in which the heart tissue was incubated). The various nitroblue tetrazolium incubating media tested are listed in Table 23. The development of the

Table 23. The NBT incubating media tested to determine the elements responsible for the maximum enzymatic macroreaction of the heart

Serial Number	NBT incubating medium	Colour of the final reaction product (F.R.P.)
1	Stock incubating solution (S.I.S.)	-
2	S.I.S. + NAD	+++
3	S.I.S. + cyanide	+
4	S.I.S. + cyanide + NAD	+++
5	S.I.S. + lactate	-
6	S.I.S. + lactate + cyanide	+
7	S.I.S. + lactate + NAD	+++
8	S.I.S. + lactate + cyanide + NAD	+++
9	S.I.S. + succinate	+
10	S.I.S. + succinate + cyanide *	+++

* Succinoxidase does not require NAD.

blue colour formazan pigment (NBT reduction) was observed. It was designated +++ when maximum; + when faint, and a - sign when no colour developed.

The following results were observed (variation in the intensity of macrostaining) using the nitroblue tetrazolium media with the additives listed in Table 23 (Figure 53) :

1 - No enzymatic macroreaction when using stock incubating solution (S.I.S.) alone. (S.I.S. is labelled "nothing " in this figure).

2 - Maximum intensity of the colour of the final product of the reaction when NAD was added to the S.I.S.

3 - Faint non-homogeneous staining of of the myocardium when cyanide was added to the S.I.S.

4 - Maximum intensity of the colour of the final reaction product when the NBT incubating medium included NAD and cyanide.

5 - No macrostaining of the heart when the NAD-linked exogenous substrate, sodium lactate, was added to the S.I.S.

6 - Faint non-homogeneous staining of the heart when the NBT incubating medium contained both lactate and cyanide.

7 - Maximum intensity of the colour of the final reaction product when the NBT incubating medium contained both lactate and NAD.

8 - Maximum intensity of the colour of the final reaction product when the NBT incubating medium contained lactate, cyanide and NAD.

9 - Faint non-homogeneous staining of the heart when the NBT incubating medium contained sodium succinate as an exogenous substrate.

10 - Maximum intensity of the macrostaining of the heart when the NBT incubating medium contained succinate and cyanide.

From the above results obtained by testing the various NBT incubating media, it can be concluded that the addition of exogenous substrate is not the limiting factor in the enzymatic macroreaction of the heart. It was only NAD which was essential: maximum enzymatic macrostaining of the heart was obtained equally with nitroblue tetrazolium incubating media containing NAD alone (2), or NAD-linked exogenous substrate (7). When the NBT stock incubating solution was used alone (1), no macroreaction was observed; but faint non-homogeneous staining occurred when cyanide was included (2), and it was apparent that NAD was the limiting factor (2, 4, 7, 8) in the dehydrogenase macroenzymatic reaction.

Succinate added to the stock NBT incubating solution resulted in a non-homogeneous staining of the heart (9), and when cyanide was added the intensity of the enzymatic macrostaining was increased (10). The role of cyanide was particularly important when non-coenzyme linked succinate dehydrogenase was examined (Figure 54).

It was apparent that nicotinamide adenine dinucleotide phosphate (NADP, coenzyme II) can be used as well as coenzyme I (NAD), in a dehydrogenase macroreaction. Thus the addition of NADP to the medium (with the heart's endogenous substrate) or the addition of NADP plus iso-citrate lead to a strong reaction in the myocardium (Figure 55A). In this case, however, addition of cyanide did not materially increase the staining intensity (Figure 55B). This indicates that NADP with its relevant dehydrogenases is an equivalent limiting factor to NAD with its dehydrogenases.

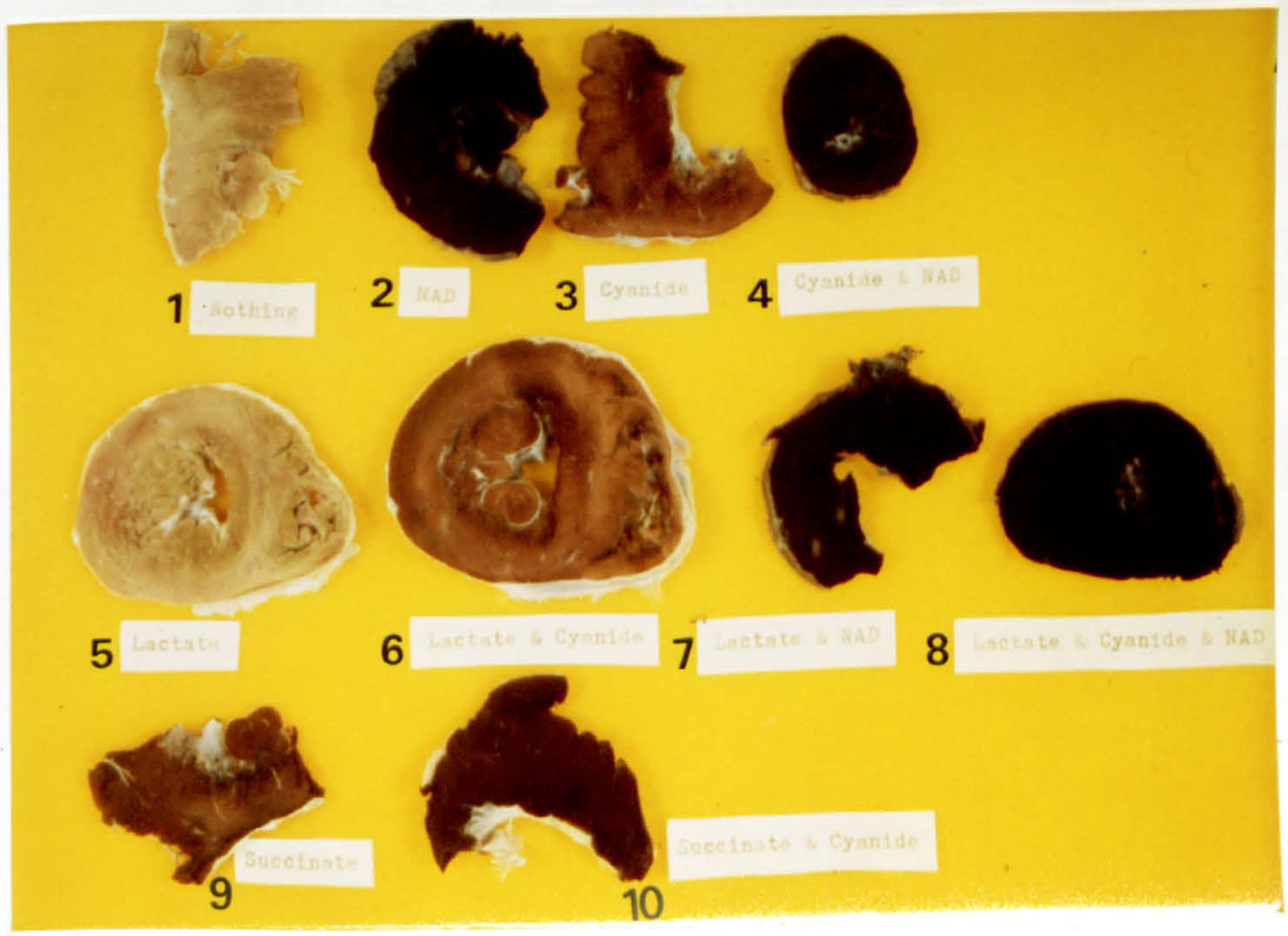


Figure 53: Variation in the intensity of macrostaining of the heart using the various NBT incubating media as labelled in the figure.
(nothing medium = stoek NBT incubating solution only or S.I.S.)

Figure 54: Succinate dehydrogenase macrostaining of two heart slices from the same normal heart. A homogeneous-dark-blue and better staining result was obtained when cyanide (CN) was added to the NBT incubating medium.

A

B

Figures 55 A and B: Figure A shows the two opposing heart slices stained with non-specific dehydrogenase with added NADP (coenzyme II, left slice), and the NADP-linked iso-citrate dehydrogenase (right slice). No difference in the macroreaction was observed when the exogenous substrate was added to the incubating medium (right slice). Figure B shows the same enzymatic macroreaction when cyanide was added to the non-specific dehydrogenase (with added NADP) incubating medium. Note the recent isolated papillary muscle infarction

B. Estimation of the effective period for the NBT dehydrogenase macroreaction using endogenous substrate with regard to the death-necropsy interval.

One of the reported difficulties with the NBT dehydrogenase macroreaction is the inadequate reaction with endogenous substrate if the death-necropsy interval exceeds six hours. Accordingly it has been previously recommended that an exogenous substrate must be added to the NBT incubating medium to compensate for the loss of the endogenous substrate (Nachlas and Shnitka, 1963). However, the results observed in this study, using NBT endogenous medium with added coenzyme and cyanide, indicate that the dehydrogenase macroreaction using endogenous substrate is independent of the death-necropsy interval. Satisfactory macro-staining of the heart was obtained with a death-necropsy interval as long as one hundred and twenty hours (Figure 56). This result emphasizes the importance of coenzyme and a respiratory chain inhibitor in the dehydrogenase macrostaining of the heart. Heart muscle normally contains substrates, coenzymes and enzymes and, of these, it would appear that coenzyme is the first to be depleted.

With regard to the effect on macrostaining of adding an exogenous substrate to the NBT incubating medium, it was found that, of the various exogenous substrates tested, β-hydroxybutyrate gave the best results. Apart from slightly accelerating the rate of the enzymatic macroreaction, it stained the heart muscle in a rich blue colour and sharply outlined areas of myocardial damage. However, the medium with endogenous substrate was more sensitive in picking up areas of early myocardial damage.

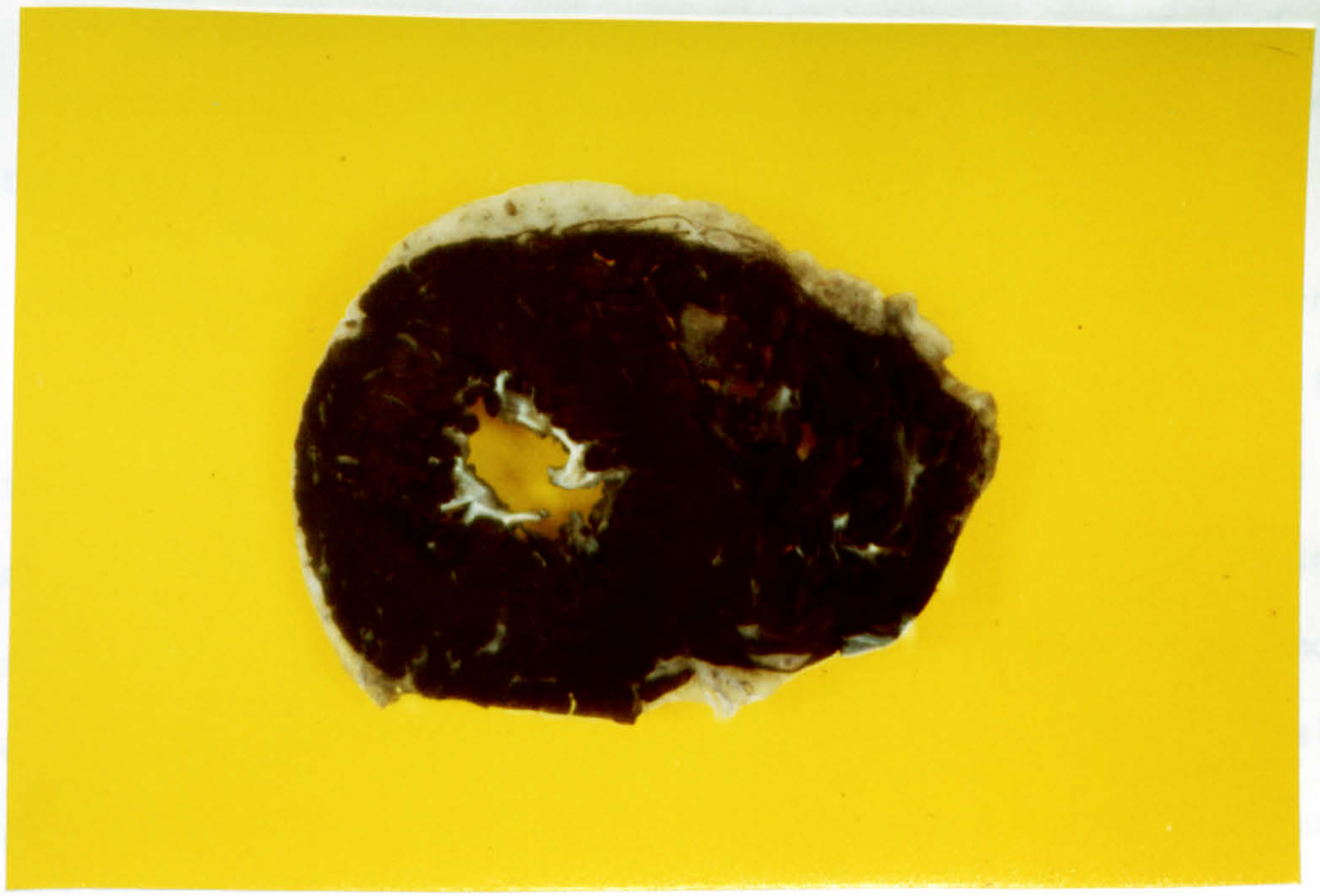


Figure 56: Normal heart slice stained with non-specific dehydrogenase NBT method with added NAD and cyanide. Homogeneous staining of the myocardium using the heart's endogenous substrate. NB:- The death-necropsy interval was one hundred and twenty hours (5 days), with storage at 4^{o}.

C. <u>The effect of postmortem autolysis on the NBT enzymatic macroreaction in regard to storage of hearts at 4° or -17° or ambient temperature</u>.

The NBT method applied in this study, with or without added substrate, was tested for the effect of postmortem autolysis on the macrostaining of the heart.

Three normal hearts were kept at ambient temperature between 18° and 25° for twenty-four to seventy-two hours. They were sampled at intervals of twenty-four hours. The NBT staining of the heart slices was not significantly diminished over the seventy-two hour period. Seven normal hearts were kept at 4° for a week, and these, likewise, showed no loss of enzyme activity. In all these cases the results of macrostaining after storage were compared with that obtained with the fresh heart slice.

Three normal hearts were kept at 4°, and two normal hearts at -17° for fourteen days. These were sampled at the seventh and the fourteenth day. The result of the NBT macrostaining of these heart slices was not significantly diminished over the fourteenth day period at 4° or at -17° (Figure 57).

Four hearts, where the enzymatic macroreaction revealed a recent myocardial infarct, were kept at 4° and at -17° for fourteen days and were sampled at the seventh and at the fourteenth day. The result of the NBT macrostaining of the slices sampled at the fourteenth day at either temperature was identical with the freshly stained heart slice from each case (Figure 58).

Figure 57: NBT macrostaining of normal heart slices with non-specific dehydrogenase. The same enzymatic macroreaction is observed in the right heart slice, which was sampled at the fourteenth day (the heart was stored at 4^{o} for two weeks), as compared with the freshly stained left heart slice.

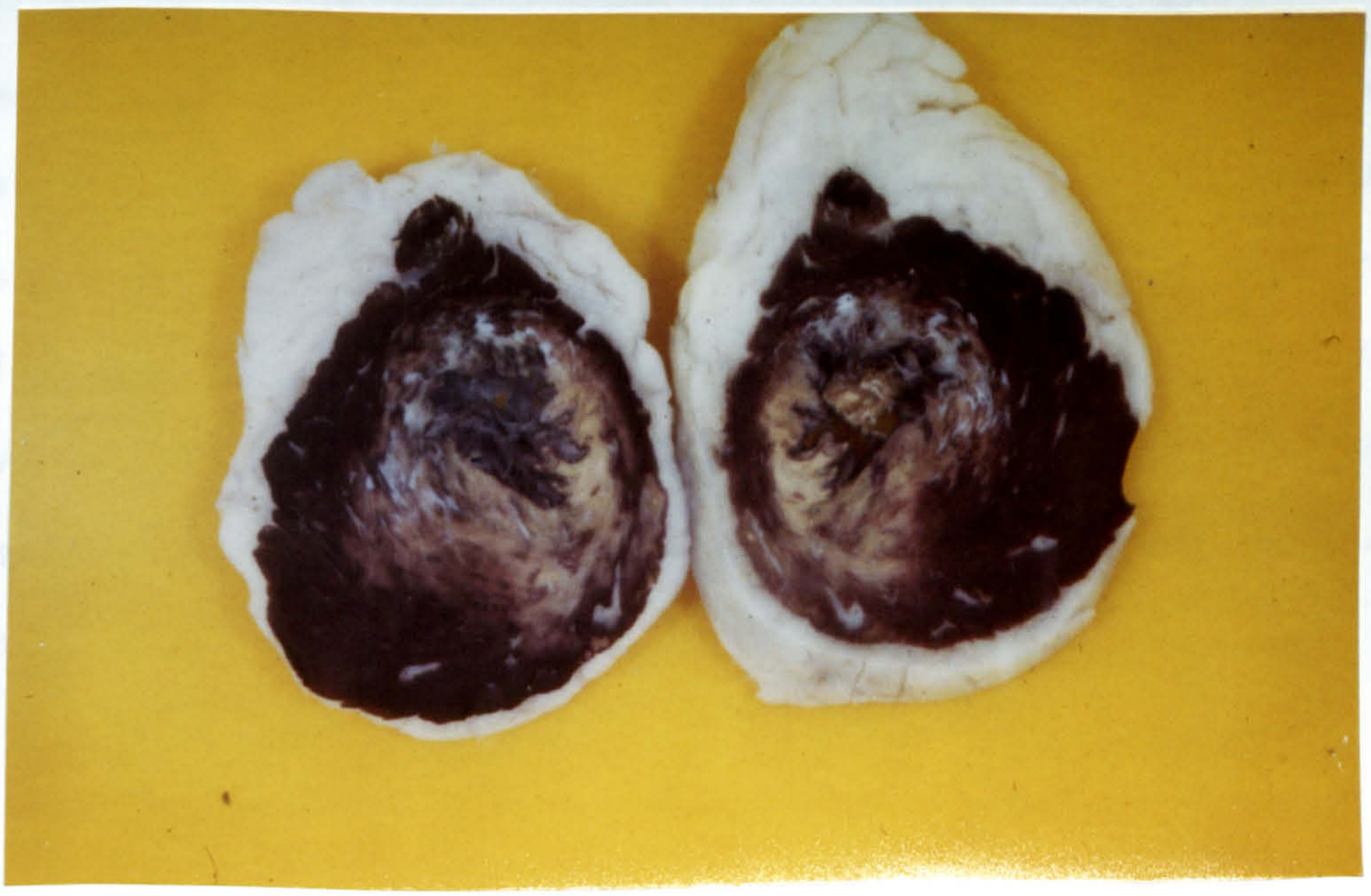


Figure 58: Non-specific dehydrogenase macrostaining of heart slices from a case of myocardial infarction. The same enzymatic macroreaction is observed in the left slice which was sampled at the fourteenth day (the heart was stored at 4° for two weeks) as compared with the freshly stained left heart slice.

D. <u>The effect on the dehydrogenase macroreaction of adding a mitochondrial protective substance (polyvinylpyrrolidone) to the NBT incubating medium</u>

Polyvinylpyrrolidone (PVP) is known to exert a protective effect on the mitochondria and, hence, increasing the accuracy of localization of dehydrogenases by providing a gel-like non-electrolyte media. However, the results obtained when adjacent heart slices of the same heart were incubated in NBT dehydrogenase incubating media with and without added PVP, showed no effect on the enzymatic macroreaction (Figure 59) and,thus polyvinylpyrrolidone did not prove particularly useful. Newer methods of improved enzymatic localisation with substrate films and semipermeable membranes have been described (Lojda, Gossrau and Schiebler, 1976), but are only applicable at the microscopic level.

E. <u>The effect on the enzymatic macroreaction of including an electron transfer mediator (phenazine methosulphate) in the NBT incubating medium</u>

Phenazine methosulphate (PMS) is frequently used in dehydrogenase histochemistry to speed up the velocity of the tetrazolium reaction by acting as an intermediate electron acceptor. PMS accepts the electrons directly from the reduced coenzymes (or flavoproteins) and transfers them directly to the tetrazolium salt without need for mediation of NADH or NADPH tetrazolium reductase , which are an integral part of the electron transfer chain and can limit the rate of transfer of electrons to the tetrazolium salt (Glenner, 1965).

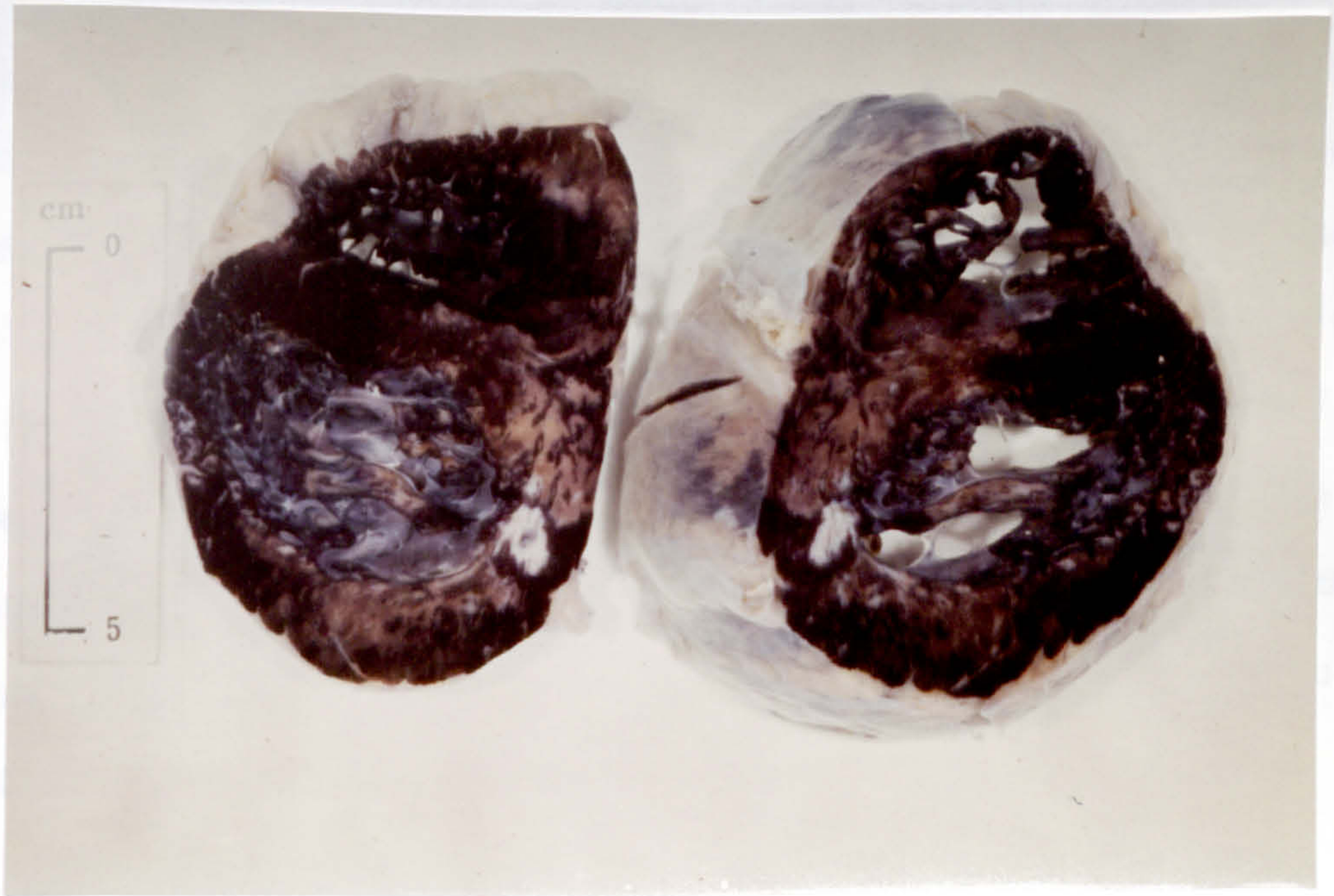


Figure 59: Two opposing heart slices from a case of myocardial infarction. Non-specific dehydrogenase macrostaining (left slice), and with added polyvinylpyrrolidone (right slice). No observed effect of polyvinyl-pyrrolidone on the enzymatic macroreaction.

The results obtained on the role of PMS in accelerating the histochemical dehydrogenase macroreaction showed that, when PMS was added to the NBT incubating medium with endogenous substrate, false macrostaining of the heart slice occurred (Figure 60). This effect was very prominent when an exogenous substrate was added to the incubating medium (Figure 61). It seems possible that PMS accelerated the transfer of electrons in solution instead of in situ, allowing soluble dehydrogenases spontaneously to reduce nitroblue tetrazolium in the incubating medium which resulted in false staining of the heart slices by the deposited formazan pigment. This is consistent with the observation that the colour of the NBT incubating medium with added PMS turned dark blue. However, when the dehydrogenase macroreaction was allowed first to take place and then PMS was added to the incubating medium in a second stage, no colour change was observed in the NBT incubating medium.

F. Dehydrogenase inhibitors

The formation of the formazan pigment in the NBT dehydrogenase macroreaction, using the heart's endogenous substrate, was found to be inhibited by p-chloromercurobenzoate, when added to the incubating medium at a concentration of 0.001 M. When the exogenous substrate β-hydroxy-butyrate was used, the enzymatic macroreaction was again inhibited by p-chloromercurobenzoate, and slightly inhibited by N-ethylmalameide (0.01 M). Malonate (0.01 M) inhibited the dehydrogenase macroreaction when succinate was used as an exogenous substrate.

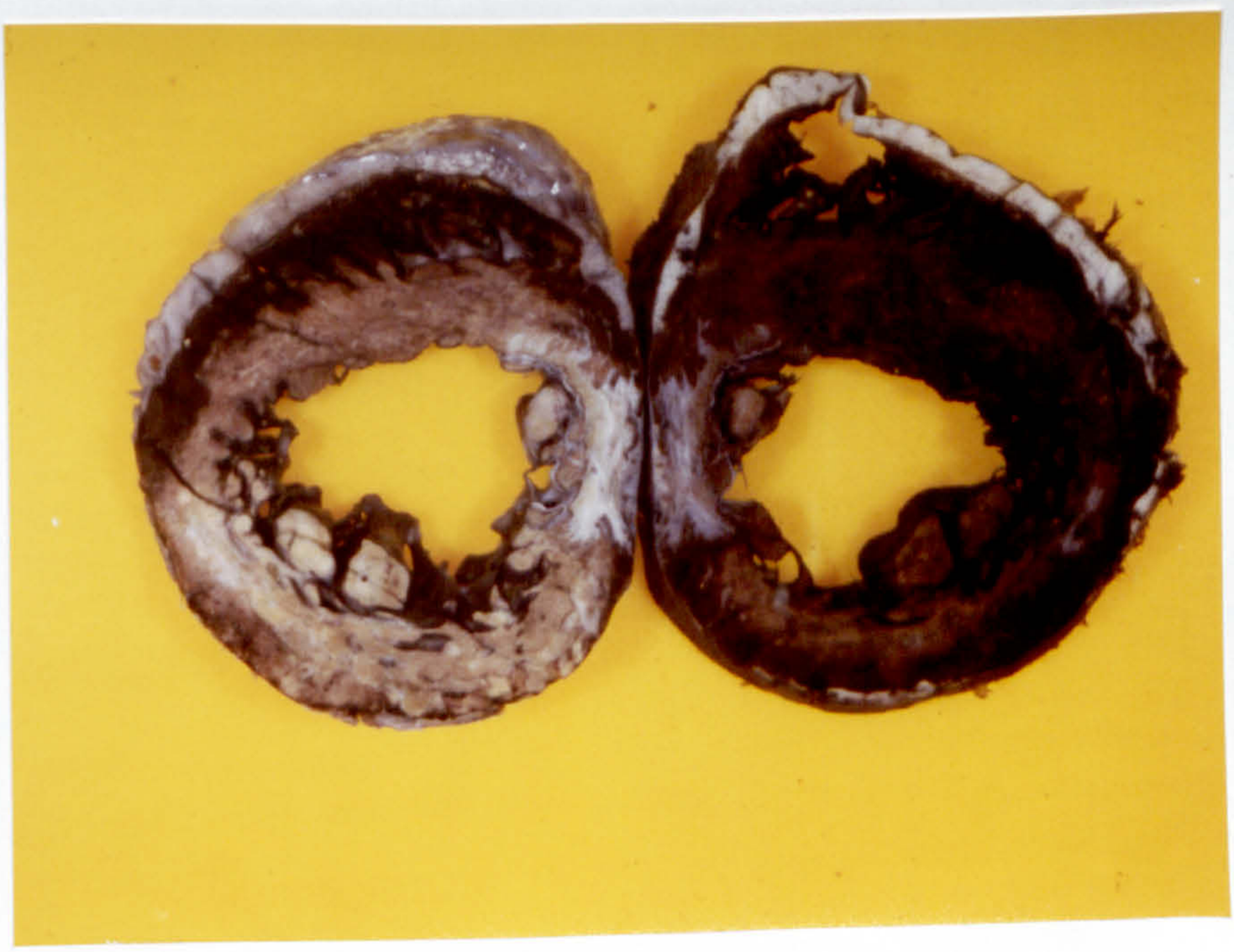


Figure 60: Two opposing heart slices from a case of myocardial infarction. Non-specific dehydrogenase macrostaining of left heart slice, and with phenazine methosulphate (PMS) added to the incubating medium with the right heart slice. False staining of the right slice largely obscures the clearly visible transmural infarct on the left.

Figure 61: Two opposing heart slices from the same case as Figure 60. β-hydroxybutyrate dehydrogenase macrostaining of right heart slice, but with phenazine methosulphate added to the incubating medium of the left slice. False staining of the left heart slice obscuring the clearly visible lesion in the right. The white fibrous scar in the posterior ventricular wall is also falsely stained.

Other inhibitors were examined. These were oxaloacetate (0.01 M), oxalate (0.01 M) and Iodoacetate (0.01 M), and were found to have no inhibitory effect on the NBT dehydrogenase macroreaction with or without added substrate (Figure 62).

G. The effect of ageing on the enzymatic macroreaction

Some material was used for studying the effect of age and of ageing on the enzyme macroreaction; the youngest case in this series was six weeks old and the oldest was eighty-seven years old. It was observed that ageing has no effect on the NBT dehydrogenase macroreaction. Satisfactory macrostaining of the heart was obtained equally in young and old subjects of both sexes.

Comparison of the applied NBT method with the original NBT method described by Nachlas and Shnitka (1963).

Nachlas and Shnitka (1963) were the first to use nitroblue tetrazolium as an oxidoreduction indicator in a dehydrogenase macroreaction for the gross identification of early myocardial infarction. The NBT method in the present study was compared with the original method. When transverse heart slices were incubated in Nachlas and Shnitka's (1963) original NBT medium, which consisted of buffered NBT stock incubating solution, the myocardium was not stained (Figure 63). These authors observed that

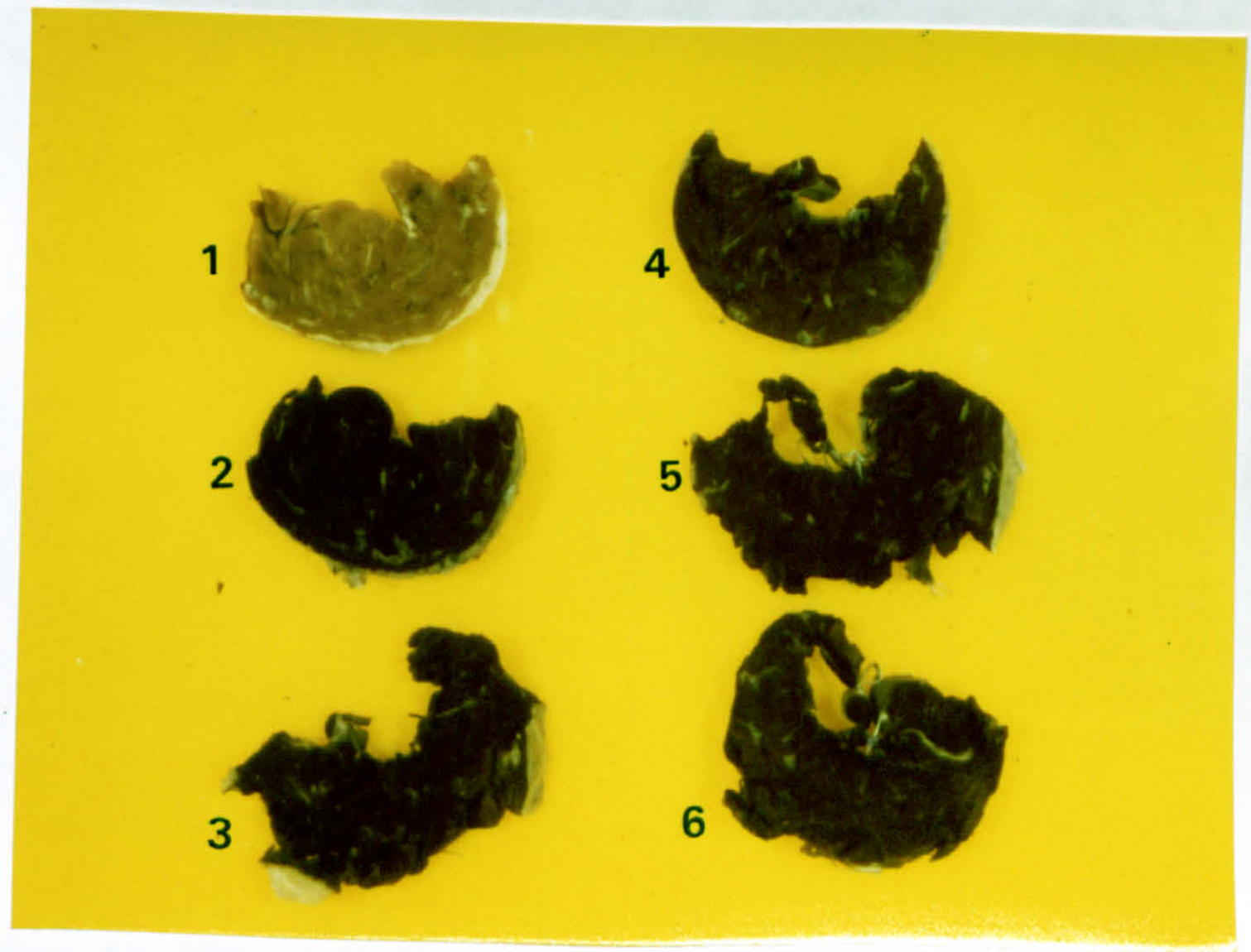


Figure 62: The effect of inhibitors on NBT non-specific dehydrogenase macroreaction of heart muscle.

1. p-chloromercurobenzoate (0.001 M)
2. malonate (0.01 M)
3. iodoacetate (0.01 M)
4. N-ethylmalameide (0.01 M)
5. oxaloacetate (0.01 M)
6. oxalate (0.01 M)

Complete inhibition by p-chloromercurobenzoate and slight inhibition by N-ethylmalameide.

Figure 63: Heart slice incubated in buffered NBT incubating solution as described by Nachlas and Shnitka (1963). No dehydrogenase macroreaction is observed.

Figure 63: Heart slice incubated in buffered NBT incubating solution as described by Nachlas and Shnitka (1963). No dehydrogenase macroreaction is observed.

endogenous substrate was lost from the heart muscle if the death-necropsy interval exceeded six hours. The results obtained in the present study showed the important role of coenzyme as the limiting factor in macroscopic staining of the heart. It was observed that when coenzyme NAD was added to Nachlas and Shnitka's buffered NBT stock incubating medium, dehydrogenase macrostaining was accomplished (Figure 64) with a slice obtained from the same heart as Figure 63. The death-necropsy interval in this case was forty-eight hours. The same effective enzymatic macrostaining of the heart using endogenous substrate medium with added coenzyme NAD was observed irrespective of the death necropsy interval. Figure 65 demonstrates two heart slices from the same heart which were incubated in Nachlas and Shnitka's nitroblue tetrazolium medium, using the heart's endogenous substrate in one case (unstained heart slice), and with added coenzyme NAD and cyanide in the other (Stained heart slice). Necropsy was performed thirty-six hours after death.

Comment on the Nature of the Enzymatic Macroreaction

The persistence of the tetrazolium "dehydrogenase" reaction when NAD is added to the incubating medium leads one to consider whether the reaction in fact depends on NADH tetrazolium reductase within the tissue. This enzyme appears to be bound to some organelle component of the cell and does not seem to be so readily solubilized as are specific dehydrogenases with the exception perhaps of the succinoxidase system. This speculation is supported by the observation above that a strong reaction is obtained within the incubating medium (with β-hydroxybutyrate

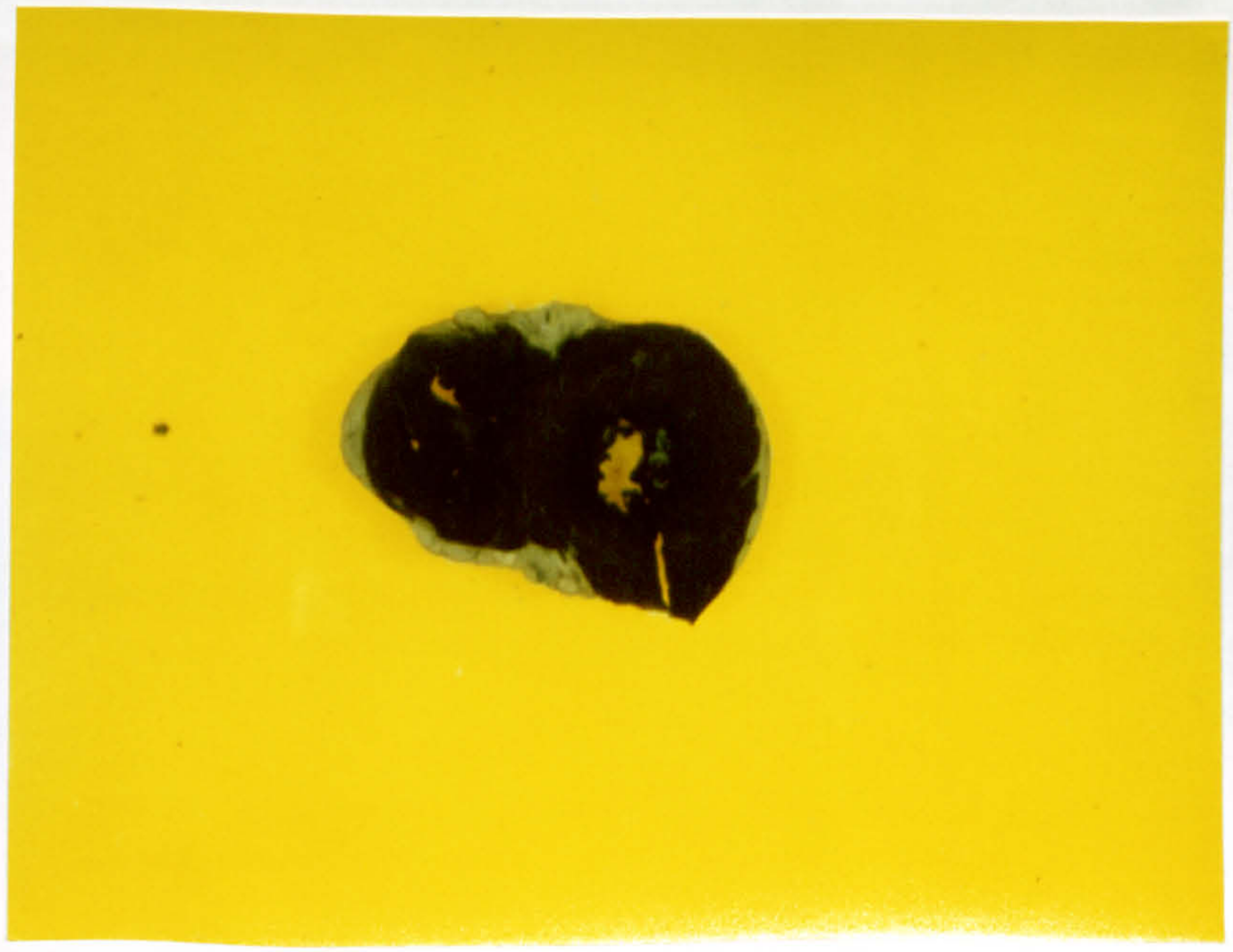


Figure 64: Heart slice from the same heart as Figure 63, incubated in the buffered NBT incubating solution described by Nachlas and Shnitka (1963), but with added coenzyme NAD. Note the dark formazan macrostaining of the heart slice.

Figure 65: Two heart slices from a suspected case of recent myocardial infarction. The right heart slice is stained after Nachlas and Shnitka's (1963) NBT method. No macroreaction is observed. The left heart slice is stained as above but with added coenzyme NAD and cyanide. The enzymatic macro-reaction has revealed a recent zonal infarction of the postero-lateral wall of the left ventricle and the papillary muscles.

as substrate) when phenazine methosulphate is added, indicating that the dehydrogenase enzyme has been leached into the medium. Furthermore, a strong "dehydrogenase" reaction is obtained with many systems where the enzyme is known to be soluble. This is most probably due to NADH (or NADPH) tetrazolium reductase in the tissue accepting electrons from dehydrogenation products within the incubating medium (Glenner, 1965; Adams, 1967). Presumably NAD accepts an electron during dehydrogenase activity in the incubating medium, so that it is reduced to NADH which is the substrate for NADH tetrazolium reductase. Finally, addition of NADH gives identical results as with addition of NAD; this further strengthens the above conclusion.

CYTOCHROME OXIDASE

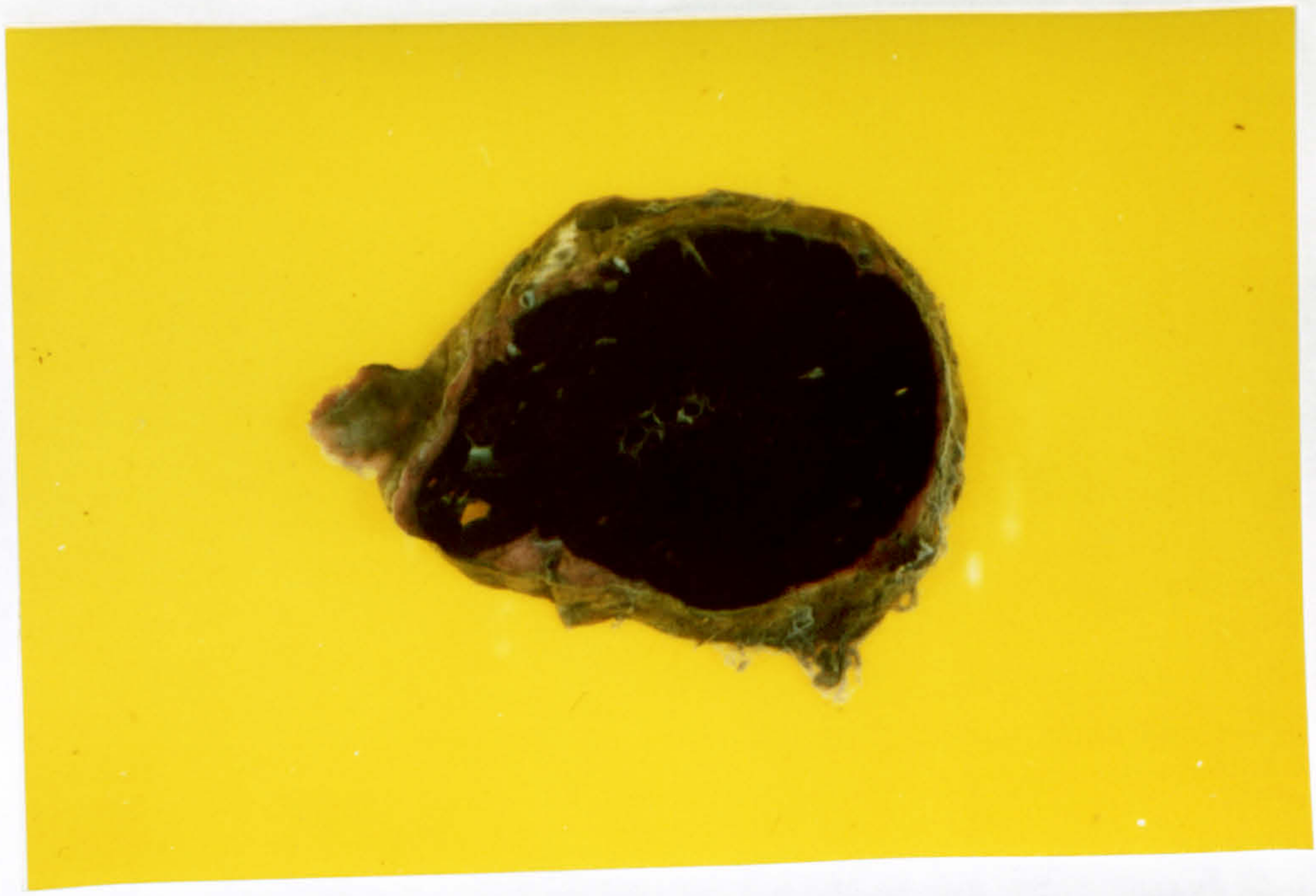
In parallel with the high activity of dehydrogenases in cardiac muscle, cytochrome oxidase activity is also conspicuous in human heart muscle, being attached to cell mitochondria.

When transversely cut heart slices were incubated in Nadi reagent for cytochrome oxidase, the heart slice was stained dark blue within two to five minutes (Figure 66A). The enzymatic macroreaction was so intense that a recent myocardial infarction, which was revealed in a heart slice stained with NBT endogenous medium, was not apparent in an opposing heart slice examined for cytochrome oxidase activity (Figure 66B). This indicates that the heart muscle is rich in cytochrome oxidase which is not affected in early myocardial infarction - thus the cytochrome oxidase macroreaction is not a sensitive indicator of early myocardial damage.

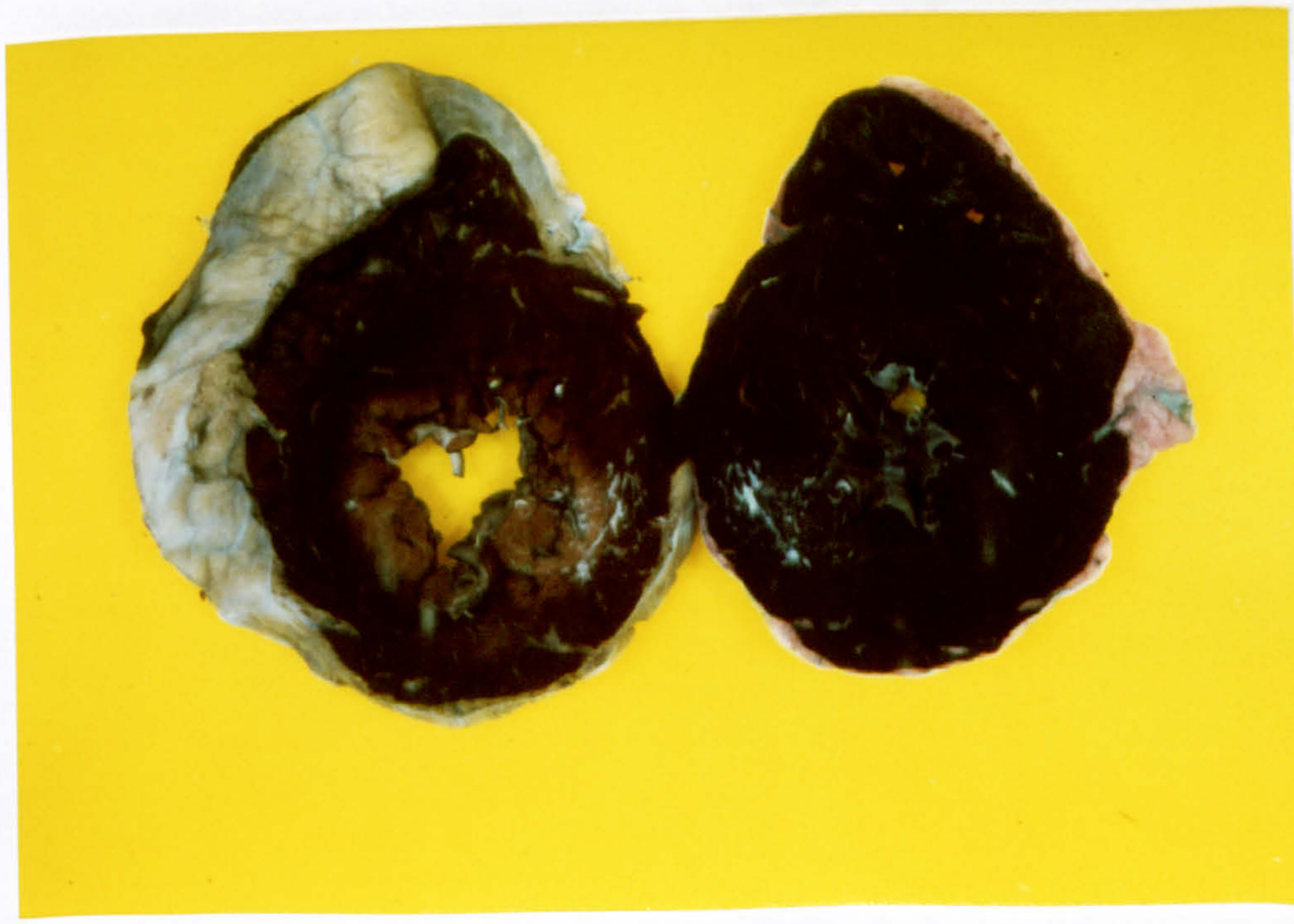
MONOAMINE OXIDASE

Monoamine oxidase activity was tested in human hearts at the macroscopic level to see whether it was of use as an indicator for early myocardial infarction. The earliest loss of enzyme activity was observed in myocardial infarction of twenty-four to forty-eight hours duration (Figure 67A). The myocardial lesion was better demonstrated in an opposing heart slice examined by the NBT test (Figure 67B).

It can be concluded that monoamine oxidase cannot be used to identify myocardial infarction under 24 hours duration.



A



B

Figures 66 A and B: Dark blue macrostaining of heart slice by cytochrome oxidase activity (A). Figure B shows two opposing heart slices from a case of myocardial infarction. A recent laminar myocardial infarct is revealed in the left heart slice by the non-specific dehydrogenase macroreaction, which is not shown by the cytochrome oxidase method applied to the adjoining heart slice.

A

B

Figures 67 A and B: Two opposing heart slices from a case of myocardial infarction revealed by a monoamine oxidase macroreaction (A), and better demonstrated by NADH tetrazolium reductase activity in the adjoining heart slice (B).

CREATINE PHOSPHOKINASE

Normal heart muscle shows high activity for creatine phosphokinase (CPK). The histochemical method available for the demonstration of CPK is a tetrazolium method, which stains normal heart muscle dark blue (Figure 68).

From the equation involved in the histochemical detection of CPK described in Chapter III (pages 109, 110), it was observed that the final pathway in the enzymatic reaction for demonstrating CPK activity is by the way of tetrazolium reductase. This was demonstrated by incubating an opposing heart slice to the one examined for CPK activity in the same incubating medium but omitting the substrate creatine phosphate: identical reactions were observed in the two heart slices (i.e. with and without the substrate creatine phosphate) (Figure 69). A similar reaction was observed when a third heart slice from the same heart was incubated in the NBT medium applied in this study, to which NAD was added (Figure 70).

Identical macroscopic reactions were obtained when opposing heart slices with myocardial damage were examined for CPK activity, using the tetrazolium incubating medium with and without the substrate creatine phosphate (Figure 71). Thus, it would seem the histochemical demonstration of CPK activity is virtually the localization of the NADPH tetrazolium reductase.

Since CPK acts through NADPH tetrazolium reductase, elimination of this stage was tried by adding phenazine methosulphate (PMS) to the incubating medium, as described in the method, to allow PMS to circumvent

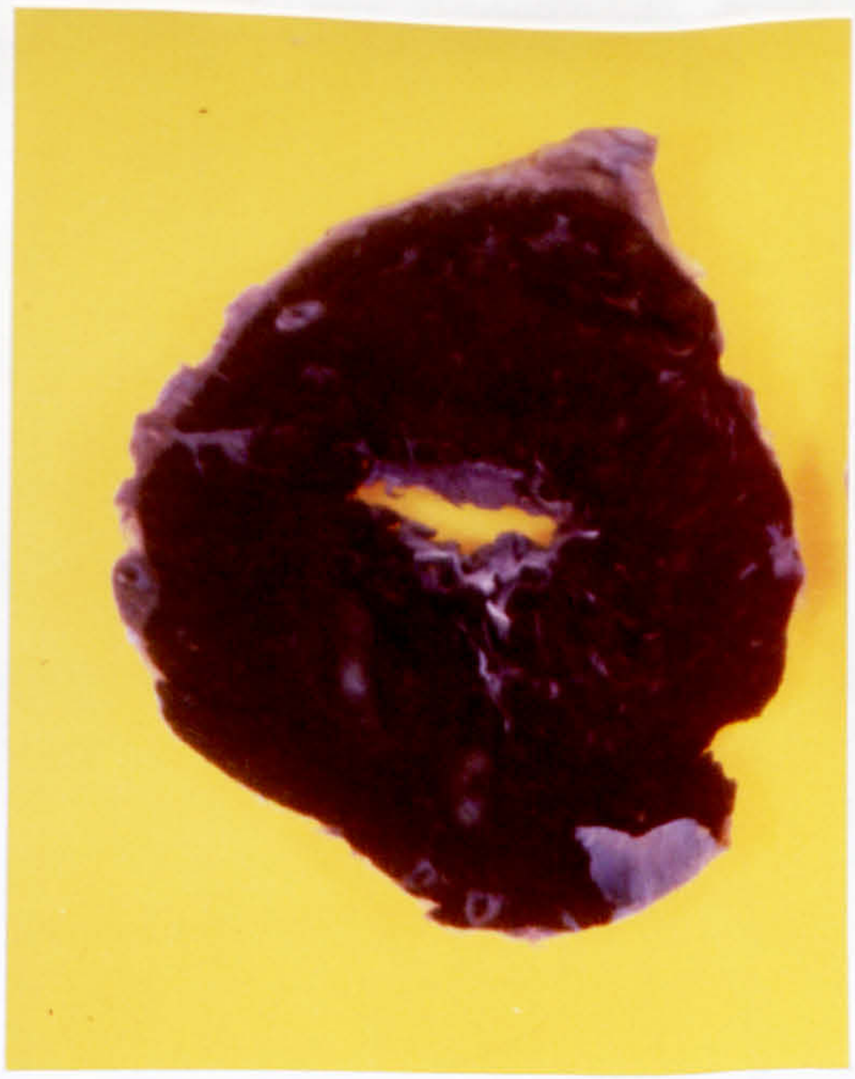


Figure 68: Dark blue staining of heart slice with creatine phosphokinase activity.

Figure 69: Two heart slices from the same heart incubated in tetrazolium incubating media for demonstration of creatine phosphokinase activity. The substrate creatine phosphate was added to the incubating medium (right heart slice), and was omitted in the other incubating medium (left heart slice). The same macro-staining reaction is observed in the two heart slices.

Figure 70: Creatine phosphokinase macrostaining of the right heart slice, and non-specific dehydrogenase macrostaining of the left heart slice (of the same heart). The same enzymatic macroreaction is observed in the two slices.

Figure 71: Two heart slices from a case of recent myocardial infarction. The same macroscopic reaction is observed in the right heart slice stained with NADPH tetrazolium reductase, and in the left heart slice with creatine phosphokinase.

NADPH tetrazolium reductase and to bring about direct reduction of the nitroblue tetrazolium salt. The result, however, was unsatisfactory as false staining of the heart slices was observed (Figure 72).

To summarize:- if the CPK method is used without PMS, then the reaction obtained seems to be due to NADPH tetrazolium reductase. However, if PMS is added tetrazolium is reduced within the medium, and artefactual non-selective staining ensues.

Other enzyme macroreactions investigated for the identification of early human myocardial infarction

The following enzymes were also examined to see whether they had a possible role in identifying early human myocardial infarction in the gross. These were:-

Myoglobin peroxidase

Phosphorylase

Glutamic-oxaloacetic transaminase

Aminopeptidase

Non-specific esterase

Acid phosphatase

The result of these enzymatic macroreactions were unsatisfactory, and they were considered unsuitable for the macroscopic diagnosis of myocardial infarction; the results are shown in Table 16 (pages 122,123). Failure to demonstrate the macroactivity of some of these enzymes, and the non-specific staining of the heart with others will be discussed in Chapter V.

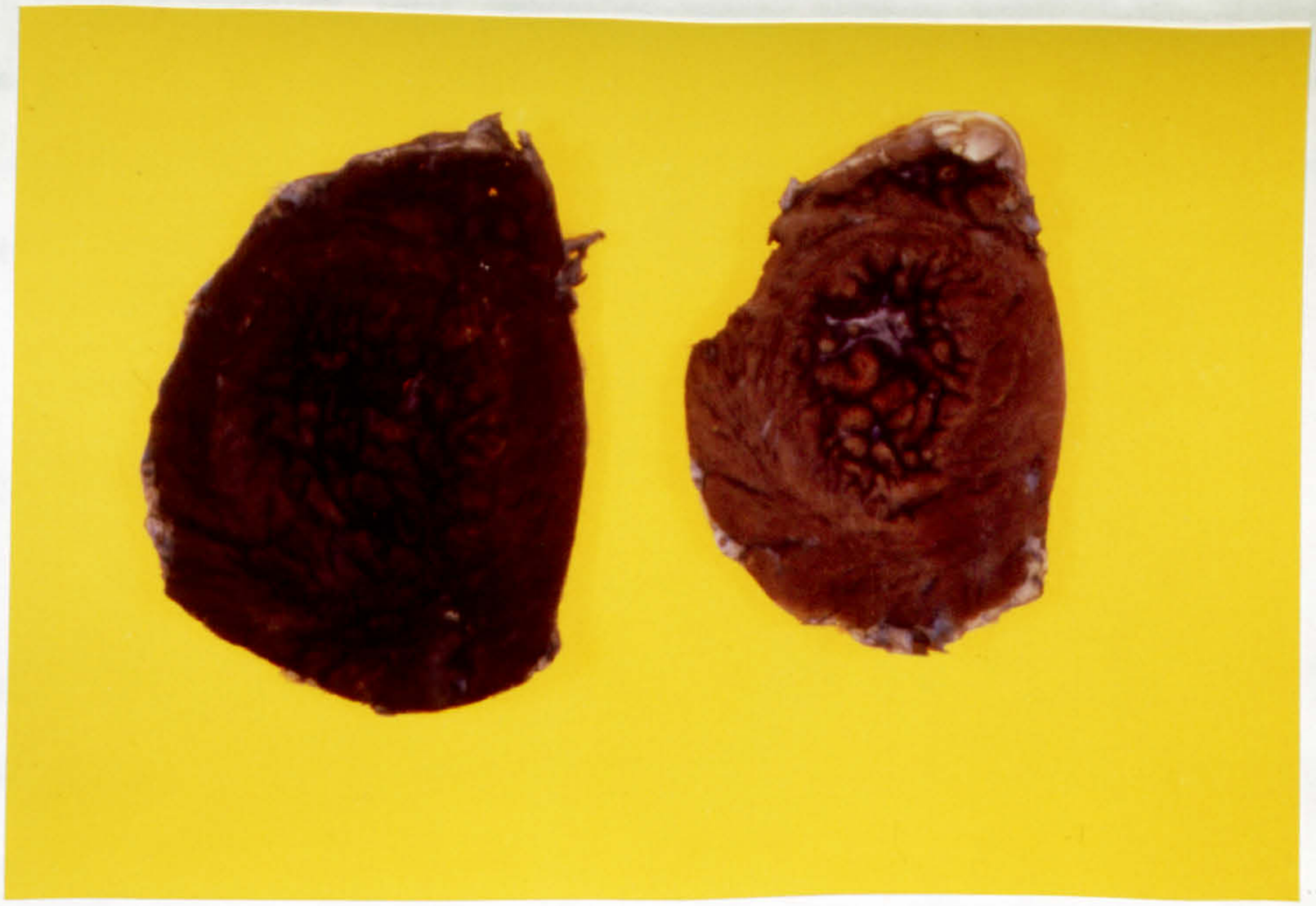


Figure 72: Phenazine methosulphate added to the incubating media for creatine phosphokinase to circumvent the tissue tetrazolium reductase system. False staining of both heart slices is observed, The substrate creatine phosphate was added to one medium (right heart slice),and was omitted in the other (left heart slice).

NON-ENZYME HISTOCHEMICAL DIAGNOSIS OF EARLY MYOCARDIAL INFARCTION

A number of non-enzyme staining techniques have been introduced for detecting early myocardial infarction under the microscope. Preliminary studies (not reported here) showed that these methods were quite unsuitable for the gross detection of infarcts. Nevertheless, the histological part of this work seems to be appropriate to mention briefly here.

PHLOXINE TARTRAZINE STAIN

The phloxine tartrazine stain was introduced by Lendrum (1947) as a general histologic stain. In this study, the method was applied to formalin-fixed, paraffin-embedded heart sections which were obtained from the hearts examined for macroscopic enzyme histochemistry, and consisted of the mirror-image slice of the infarct, as revealed by the enzymatic macroreaction, and of the adjacent normal myocardium.

Selective staining of myocardial fibres was observed. Ischaemic myocardial fibres stained bright red in contrast to normal fibres which stained yellow (Figures 73, 74, 75).

The affinity of the ischaemic fibres to the stain was striking, and dramatic long before myocardial fibres show any histologic evidence of necrosis (Figure 76), and did not appear to vary with the age of the infarct. It was observed that the under one hour infarct as illustrated in Figure 76, and the five hours infarct (Figure 77) showed the same affinity to the stain. However, it should be noted that less intensely

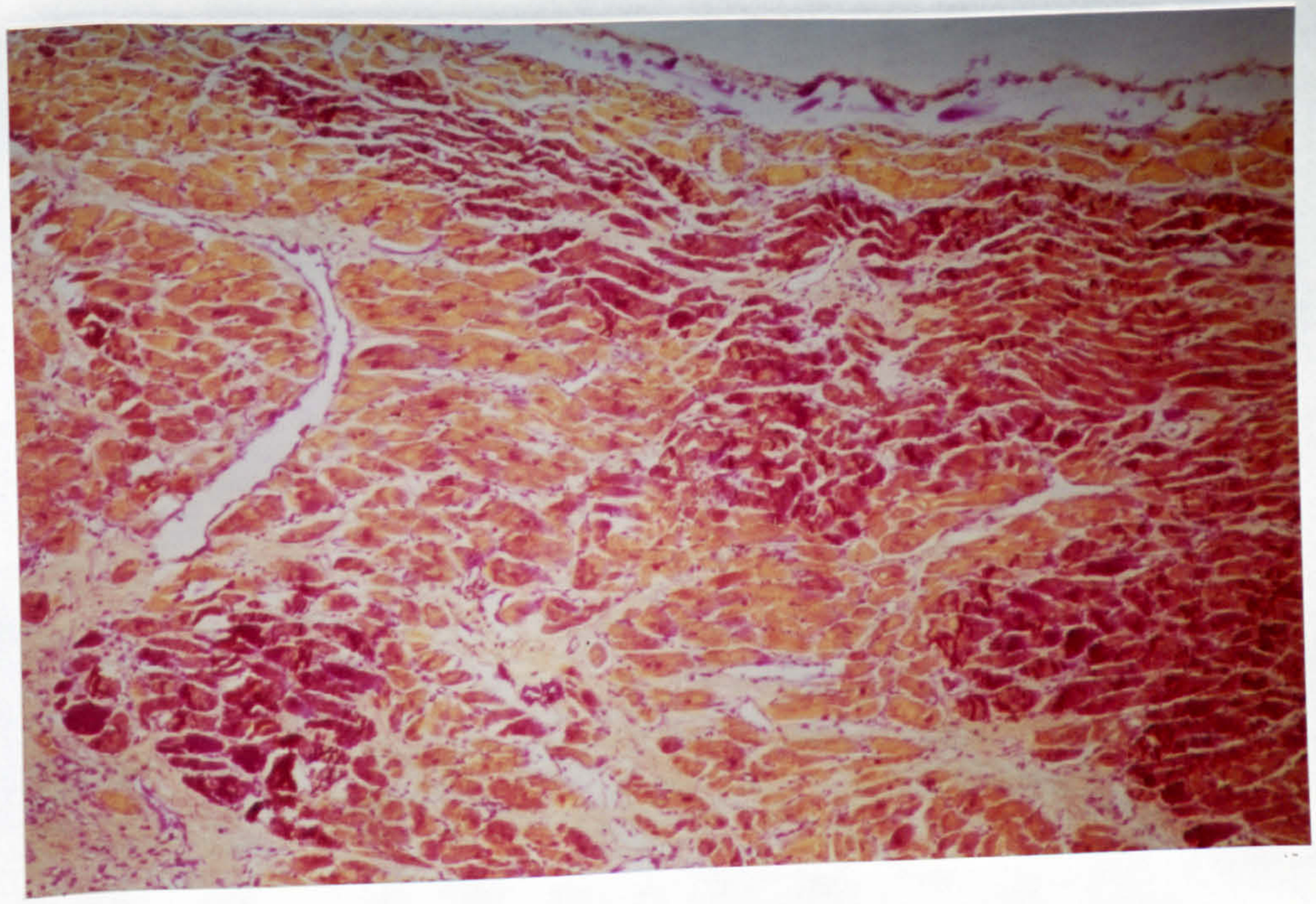


Figure 73: Heart section from a case of myocardial infarction of clinical age under one hour. Selective staining of myocardial fibres is observed:- ischaemic fibres are stained bright red, while normal fibres are stained yellow. Note the yellow staining of the narrow zone of viable myocardial fibres immediately beneath the endocardium (top). Phloxine tartrazine, X 232.

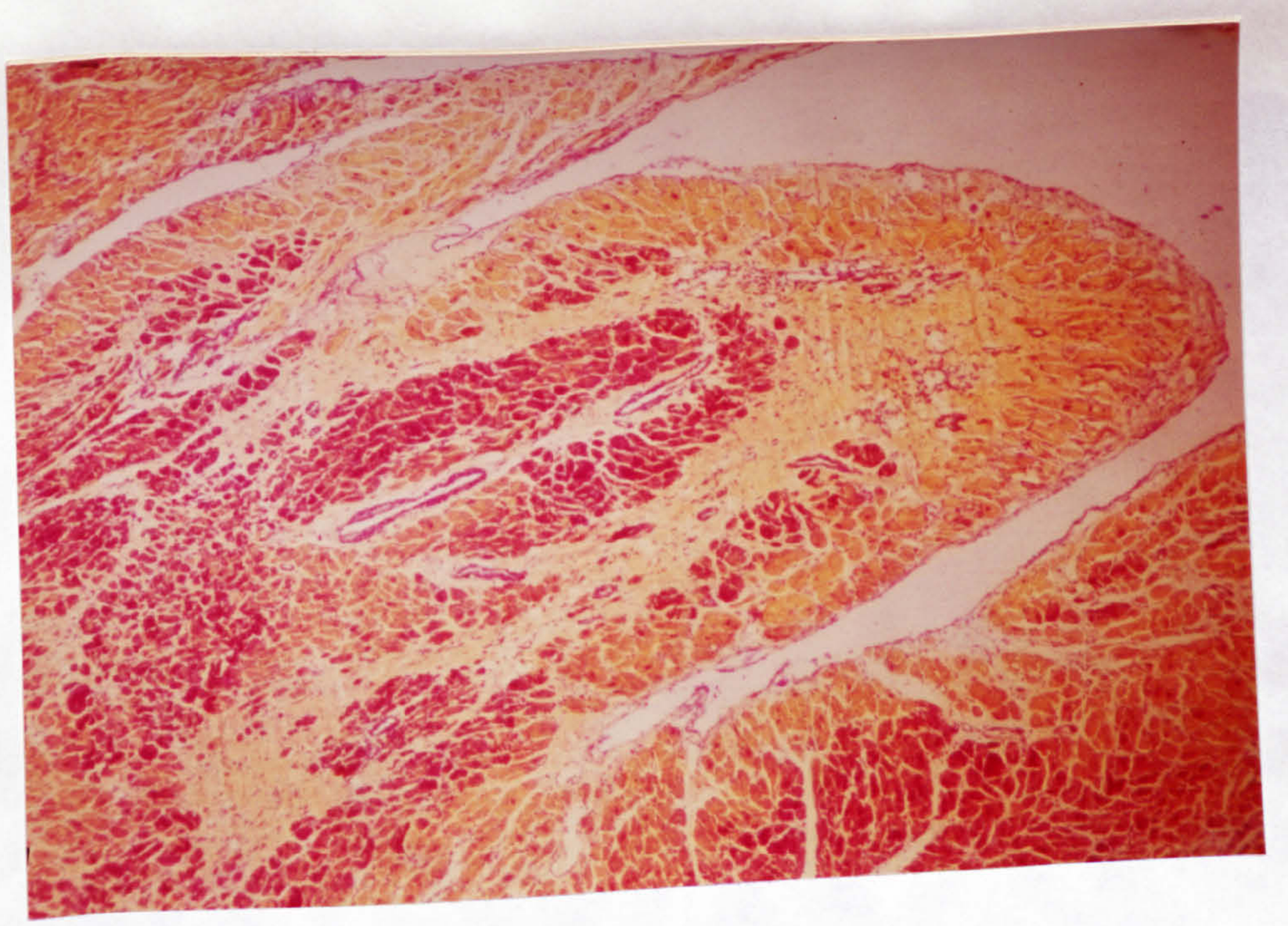


Figure 74: Section of papillary muscle from a case of myocardial infarction ofclinical age 1½ hours. The infarction of the papillary muscles appeared to be contemporary with that of the body of the myocardium. Ischaemic fibres are stained bright red. The viable band of subendocardial fibres are stained yellow, as are areas of fibrous tissue. Phloxine tartrazine, X 125.

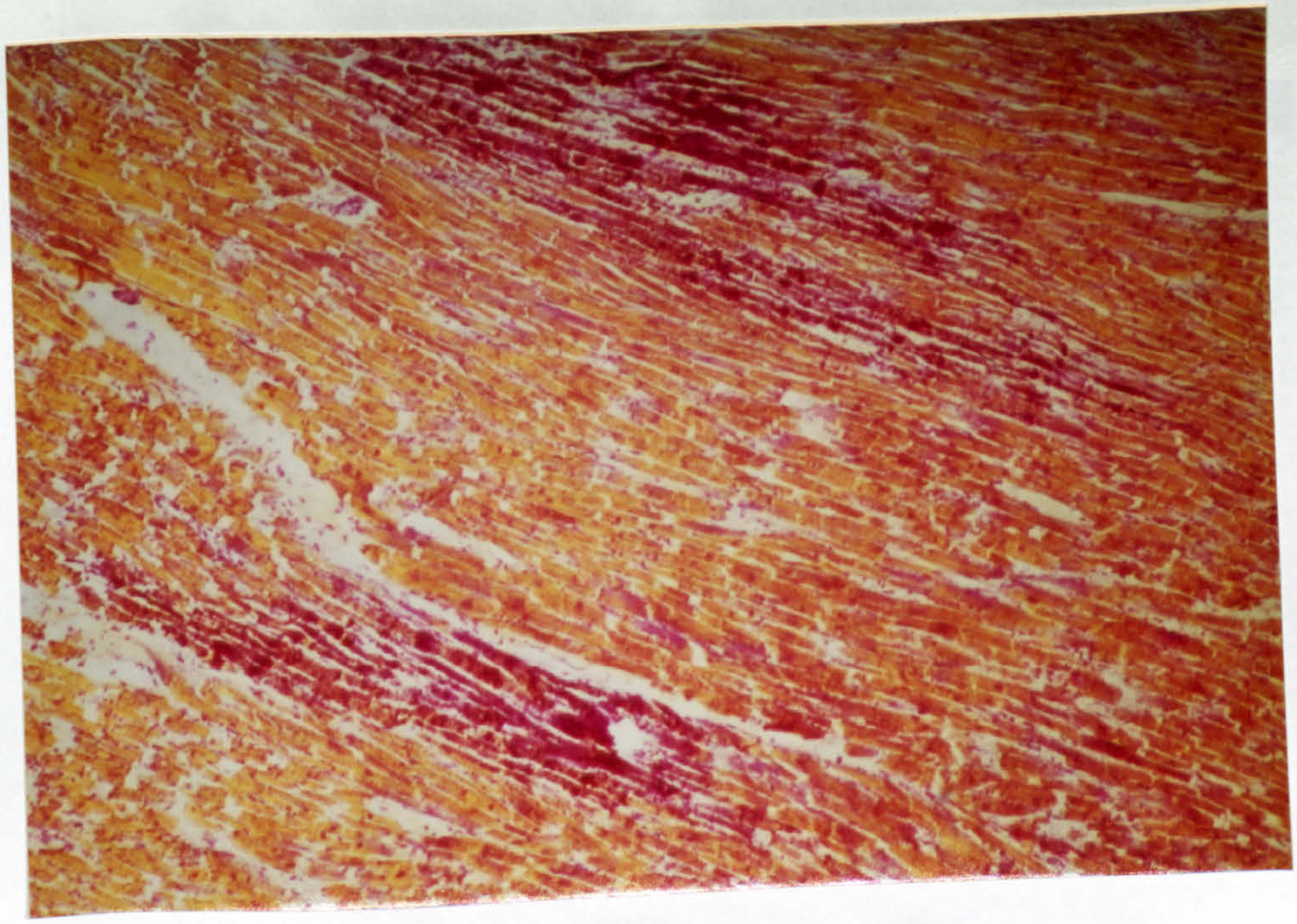


Figure 75: Heart section from a case of myocardial infarction of 4½ hours clinical age. Bright red staining of infarcted myocardial fibres, and yellow staining of normal fibres. Phloxine tartrazine, X 125.

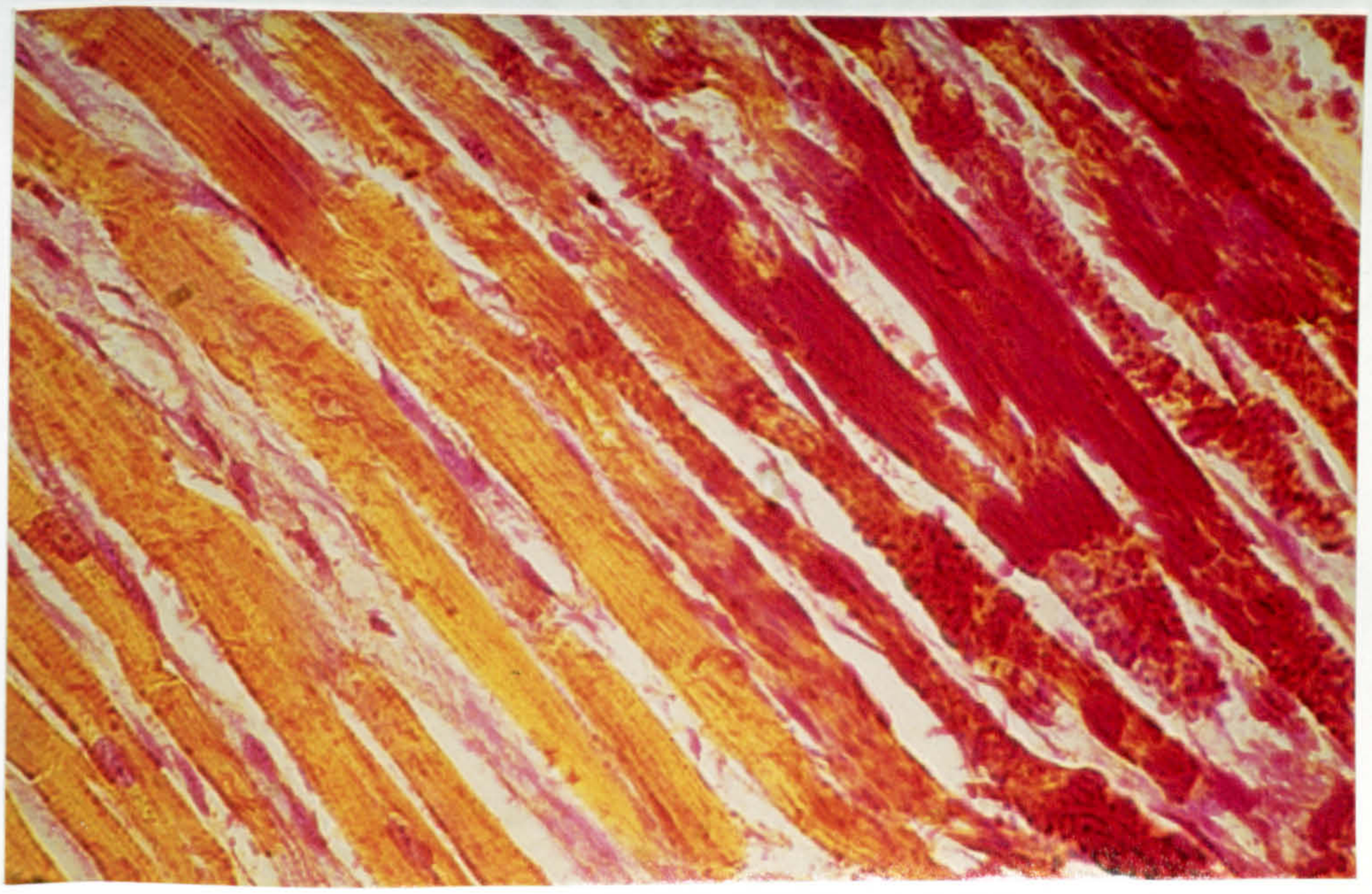


Figure 76: Phloxine tartrazine staining of a heart section from a case of myocardial infarction of clinical age under one hour. Ischaemic myocardial fibres are stained bright red and some myocardial cells show diffuse granularity. Normal myocardial fibres are stained yellow , X 310.

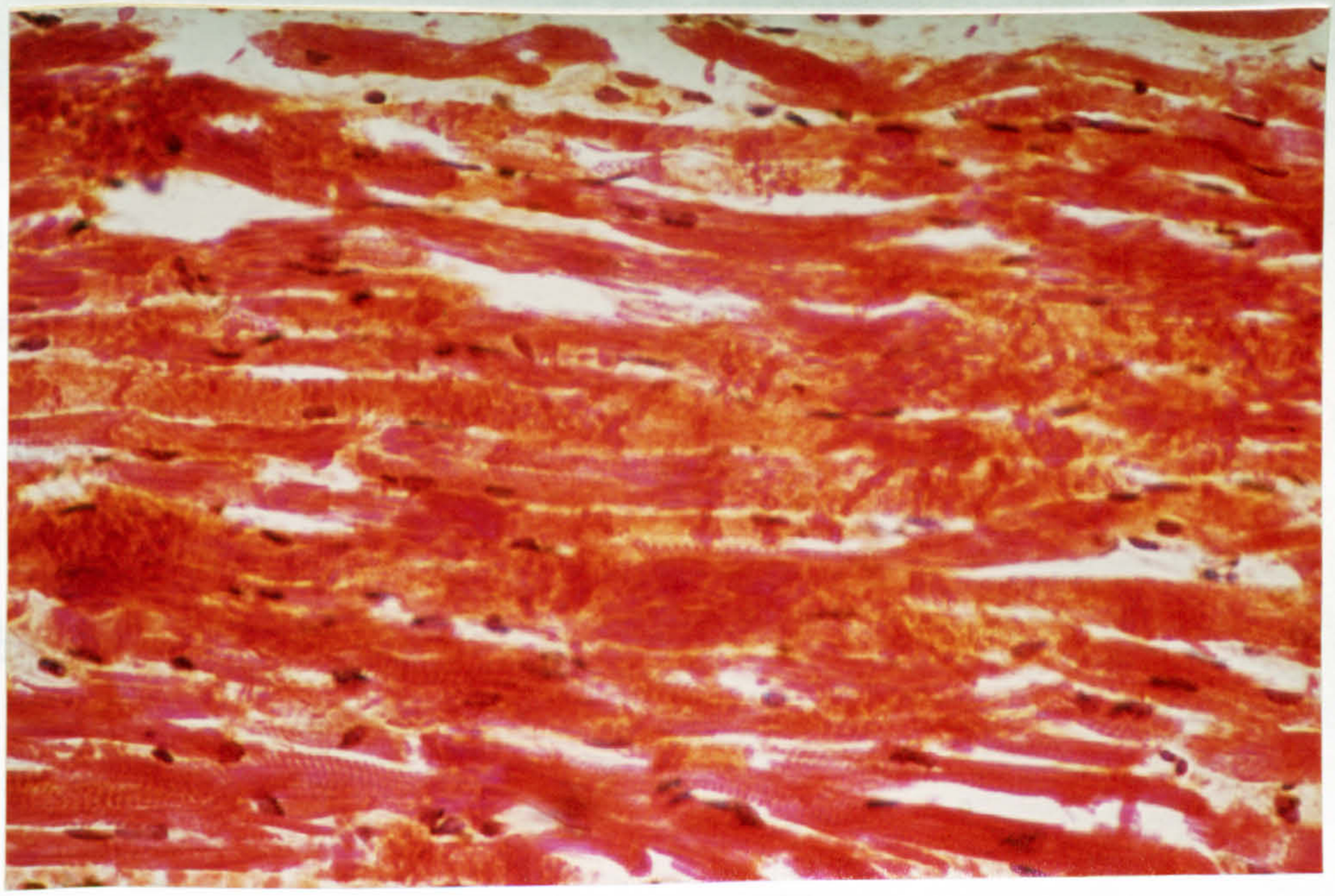


Figure 77: Phloxine tartrazine stain of a heart section from a case of myocardial infarction of 5 hours clinical age. Infarcted myocardial fibres are stained bright red. Some fibres are swollen, granular and show structural disorganization and disruption of the myofibrillar pattern. By contrast, other infarcted fibres at the bottom of the figure do not show structural changes and the Z-bands are of regular arrangement, X 310.

bright red stained necrotic fibre were seen in old myocardial infarction (Figures 78, 79).

Postmortem autolysis did not appear to alter the results of staining of the normal or infarcted myocardial fibres. No false positive or false negative staining was observed up to a death necropsy interval of ninety-six hours (Figure 80), and when hearts were allowed to autolyse at ambient temperature between 18° and 25° for three days, or when stored at 4° and -17° for two weeks (Figure 81).

HAEMATOXYLIN-BASIC FUCHSIN-PICRIC ACID (HBFP) STAIN

This stain was introduced by Lie, Holley, Kampa and Titus (1971), for the histochemical diagnosis of myocardial ischaemia. Normal myocardial fibres stain light brown, while ischaemic fibres stain crimson red (fuchsinorrhagia). When the method was applied in this study, inconsistent and unreliable results were obtained as the method depends particularly on the extent to which the sections are differentiated. Under-differentiation provided false positive staining and vice versa (Figure 82A and B).

ACID FUCHSIN STAIN

The method was designed by Selye (1958) to evaluate toxic cardiac necrosis in experimental animals, and was applied by Poley, Fobes and Hall (1964) for the histochemical identification of early myocardial

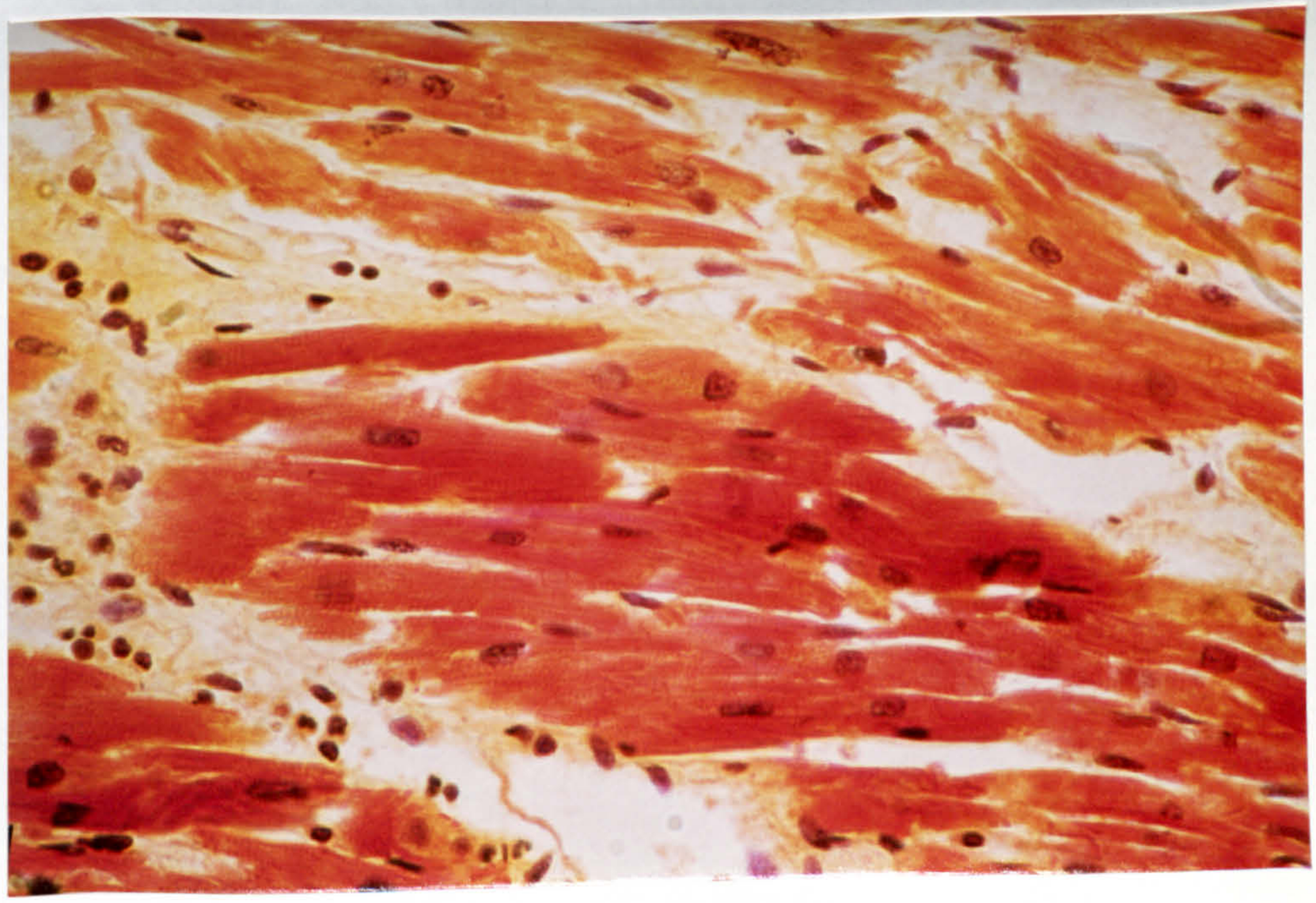
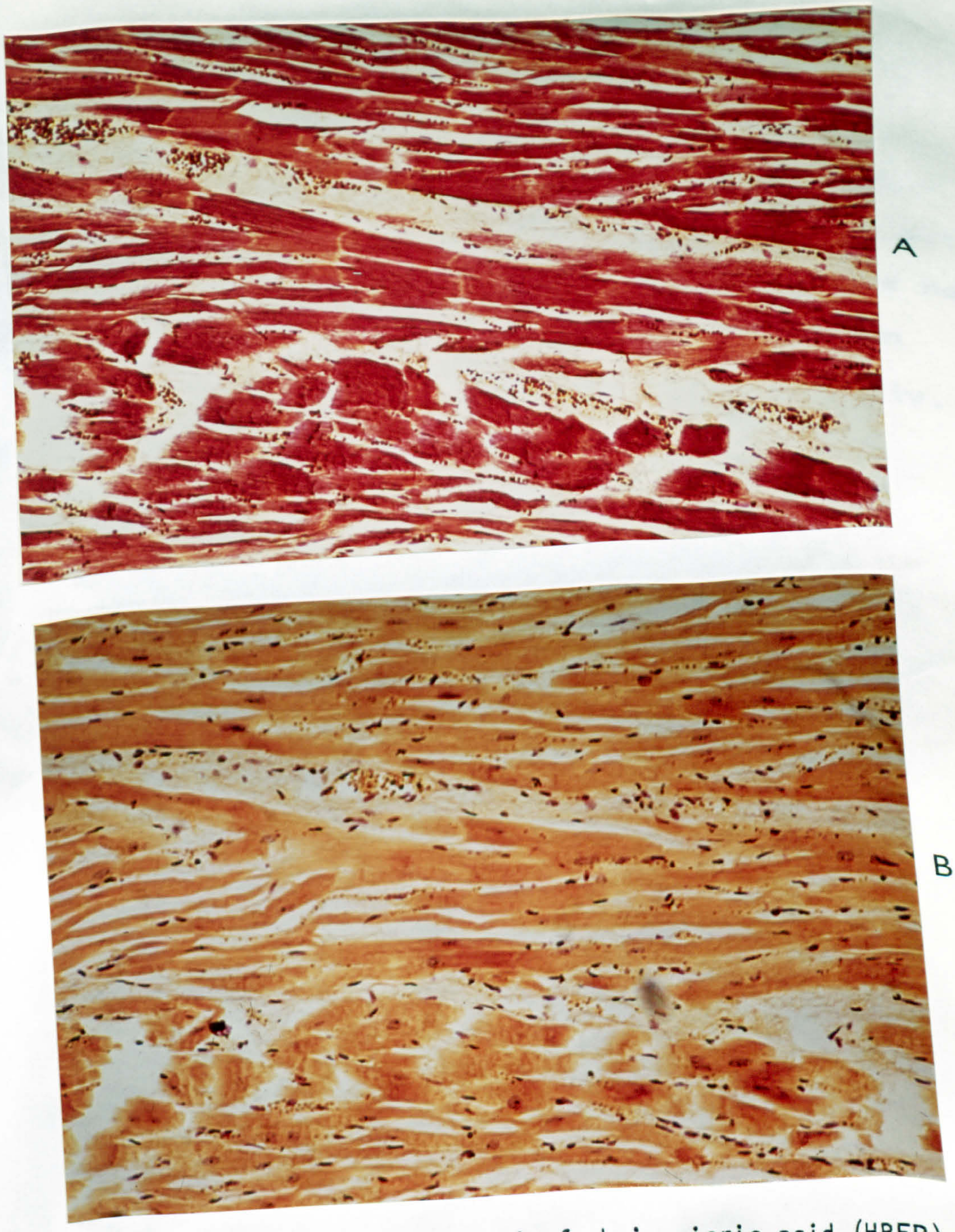


Figure 78: Phloxine tartrazine staining of a heart section from a case of myocardial infarction of 14 hours clinical age. Note the early neutrophil polymorph infiltration. The disorganized infarcted myocardial fibres at the top of the figure show less affinity for phloxine than the swollen turgid infarcted fibres at the middle. The latter, however, have better retained their myofibrillar structure, X 310.

Figure 79: Heart section from a case of myocardial infarction of 24 hours clinical age. Infarcted myocardial fibres with clear necrosis and absent nuclei show a somewhat decreased affinity for phloxine. Phloxine tartrazine, X 232.

Figure 80: Yellow staining of normal myocardial fibres with phloxine tartrazine. Death-necropsy interval 96 hours, X 232.

Figure 81: Section of normal heart autolysed at ambient temperature for 3 days. Yellow staining of myocardial fibres. Phloxine tartrazine, X 232.



A

B

Figures 82 A and B: Haematoxylin-basic fuchsin-picric acid (HBFP) stain from a case of myocardial infarction of 5 hours clinical age. The crimson-red fuchsin staining (fuchsinorrhagia) of muscle fibres in section A is absent from the serial section B, X 310.

infarction. Normal myocardial fibres stain blue green and ischaemic myocardial fibres stain dark red (fuchsinophilia).

The staining results obtained in this study with acid fuchsin were more consistent than with HBFP stain. However, the method did not provide a sharp differential staining between the normal and the necrotic fibres (Figure 83), and was observed to be clearly positive when structural changes of myocardial fibres were seen microscopically.

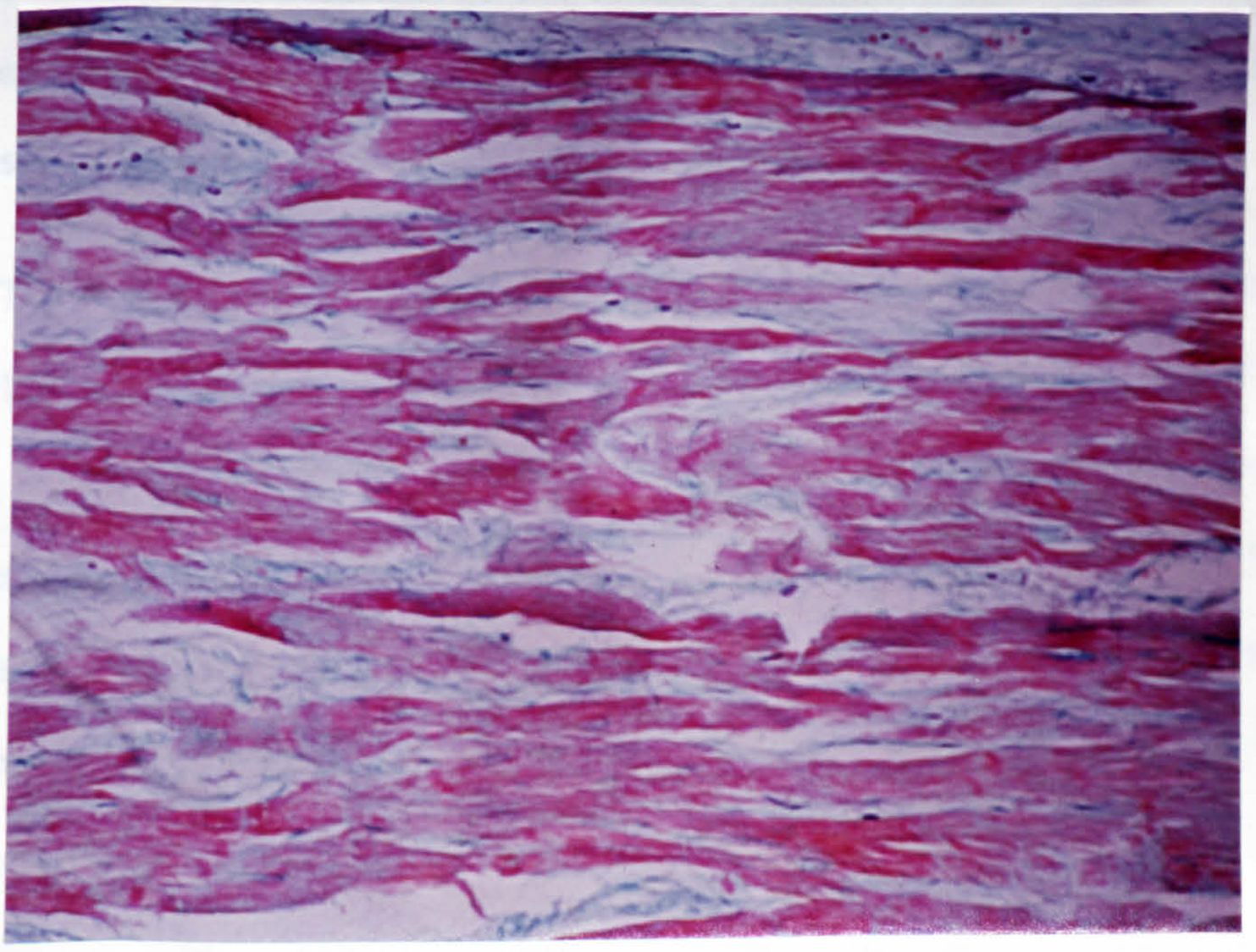


Figure 83: Acid fuchsin stain of a heart section from a case of myocardial infarction of 4½ hours clinical age. Differential staining between normal myocardial fibres (blue green), and infarcted fibres (dark red) is not clearly demonstrated, X 310.

EVALUATION OF THE WAVY FIBRE AS A MICROSCOPIC INDEX OF MYOCARDIAL INFARCTION

In the early phase of this work much importance was attached to the wavy fibre (Bouchardy and Majno, 1971/1972, 1974) as a microscopic indicator of early myocardial infarction. However, as will be seen, it became apparent that it is not a specific feature of ischaemic heart disease.

To determine the importance of the wavy fibre, the myocardium in normal and infarcted human heart was examined microscopically to see how often wavy fibres are present in both conditions. Twenty-eight normal hearts from the negative controls used in the present study were obtained from subjects with no clinical history of angina, infarction or hypertension. None of them showed evidence at autopsy of myocardial infarction or of coronary occlusion by thrombosis or stenosing atheroma; in none of them was there a severe grade of coronary atherosclerosis. A heart was also obtained from an infant aged six weeks who had died with no disease of the cardiovascular system. Thirty-one hearts were obtained from subjects in whom postmortem and microscopical evidence of myocardial infarction had been obtained. All hearts were examined blind by a second observer, with the areas of infarction or 'dummy areas' blotted out with black ink.

The results of the microscopic examinations showed that wavy fibres were present in about half the normal and half of the infarcted human hearts (Table 24). They were even present in the heart of the six week old infant (Figure 84), and were also seen in the young (Figure 85), and old subjects (Figure 86). These wavy fibres were

Table 24: Incidence of wavy fibre in normal and infarcted human myocardium

Number of wavy fibres	Normal hearts	Infarcted hearts
None or equivocal	13	16
Moderate	9	7
Many	6	8
Total	28	31

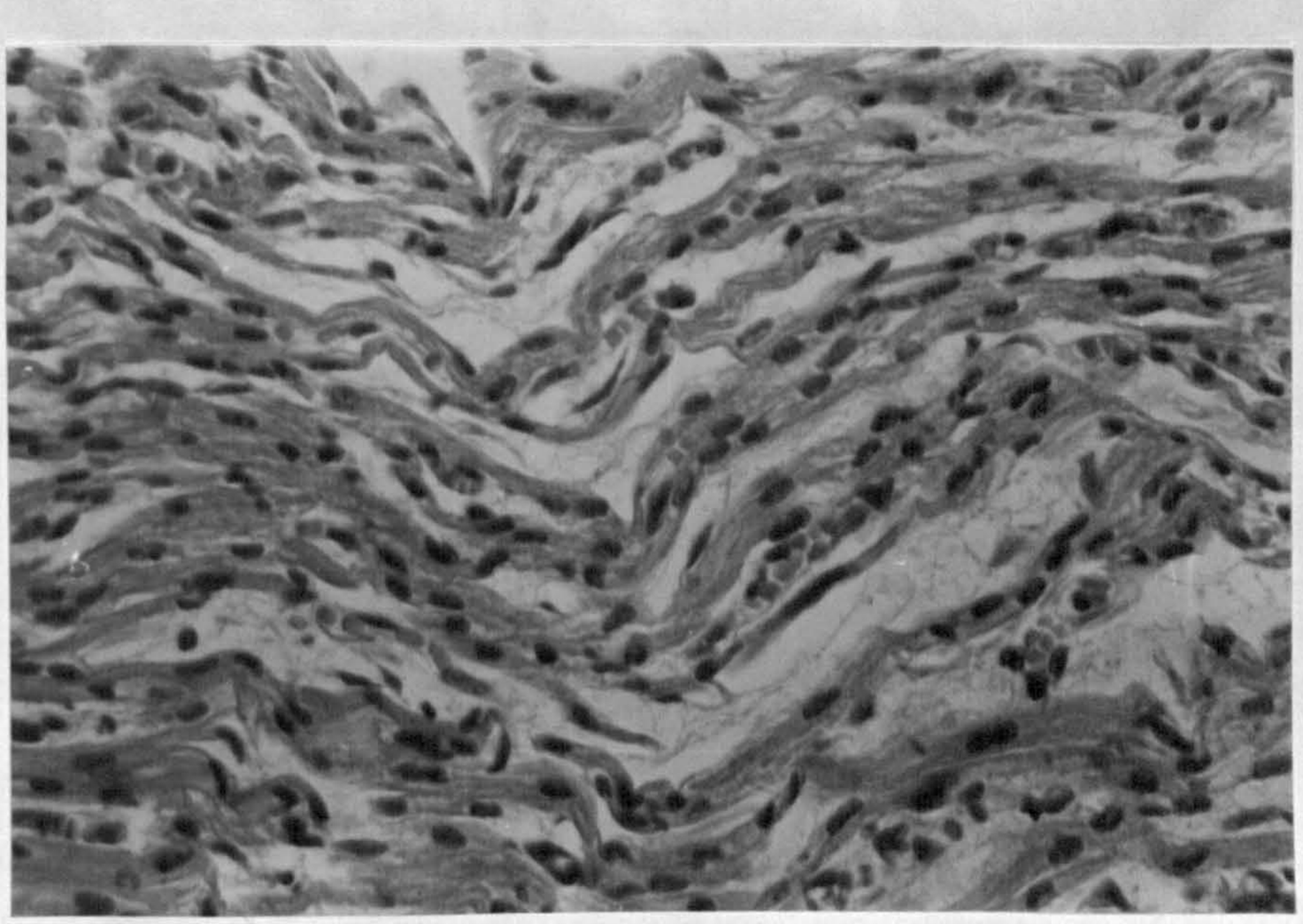


Figure 84: Wavy fibres in the normal heart of a 6-week-old infant (cot death). Haematoxylin and eosin, X 240.

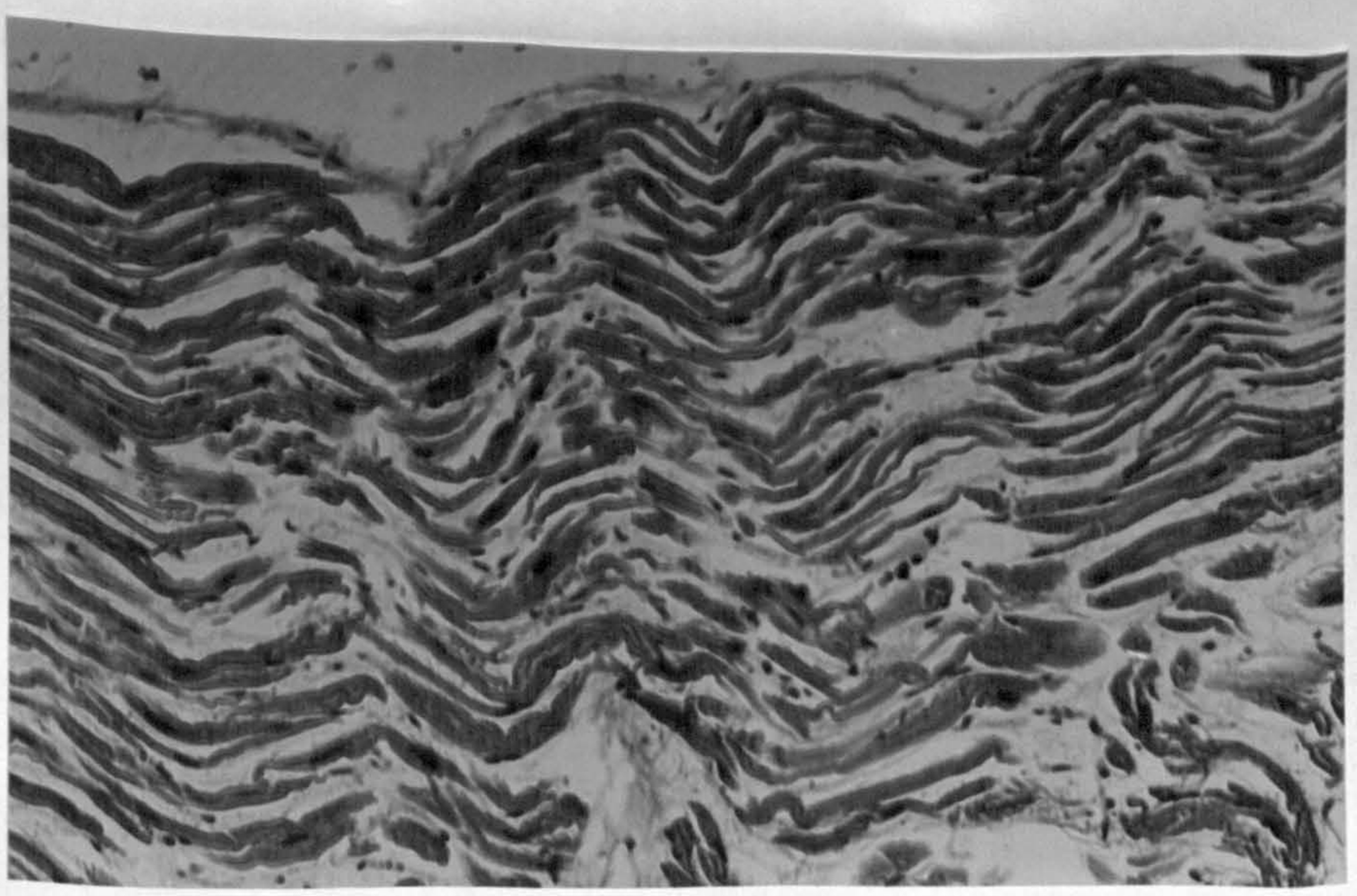


Figure 85: Wavy fibres in the normal heart of a 33-year-old man who was killed in a motor car accident. Haematoxylin and eosin, X 125.

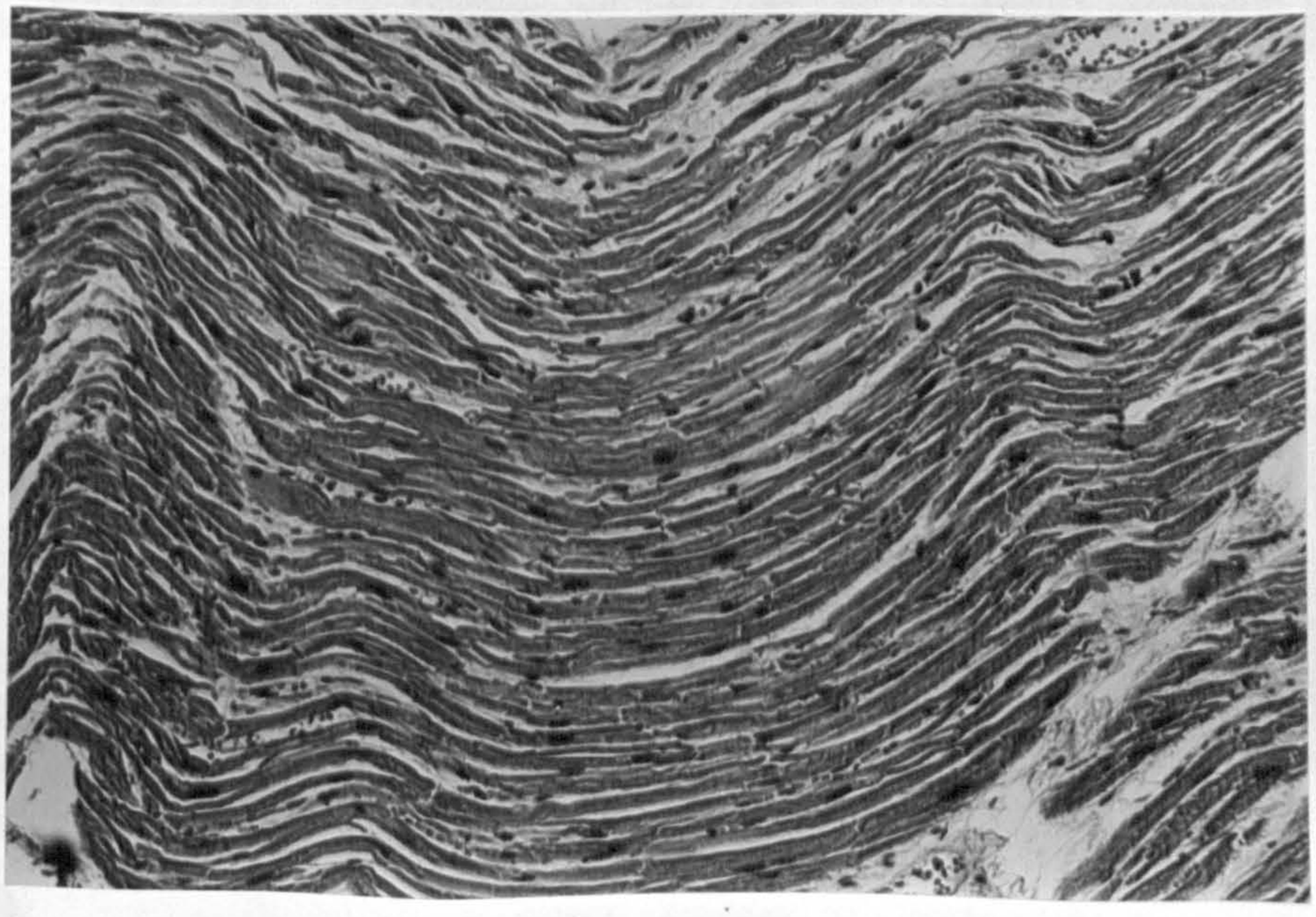


Figure 86: Wavy fibres in the normal heart of a 72-year-old woman who died of broncho-pneumonia. Haematoxylin and eosin, X 125.

indistinguishable from those at the margin of myocardial infarcts (Figures 87, 88, 89). The wavy fibres were also seen in three autolysed normal hearts (Figure 90). Thus, it would appear that the wavy fibres are not specific features of acute myocardial infarction and cannot be used as an early morphologic sign of the lesion.

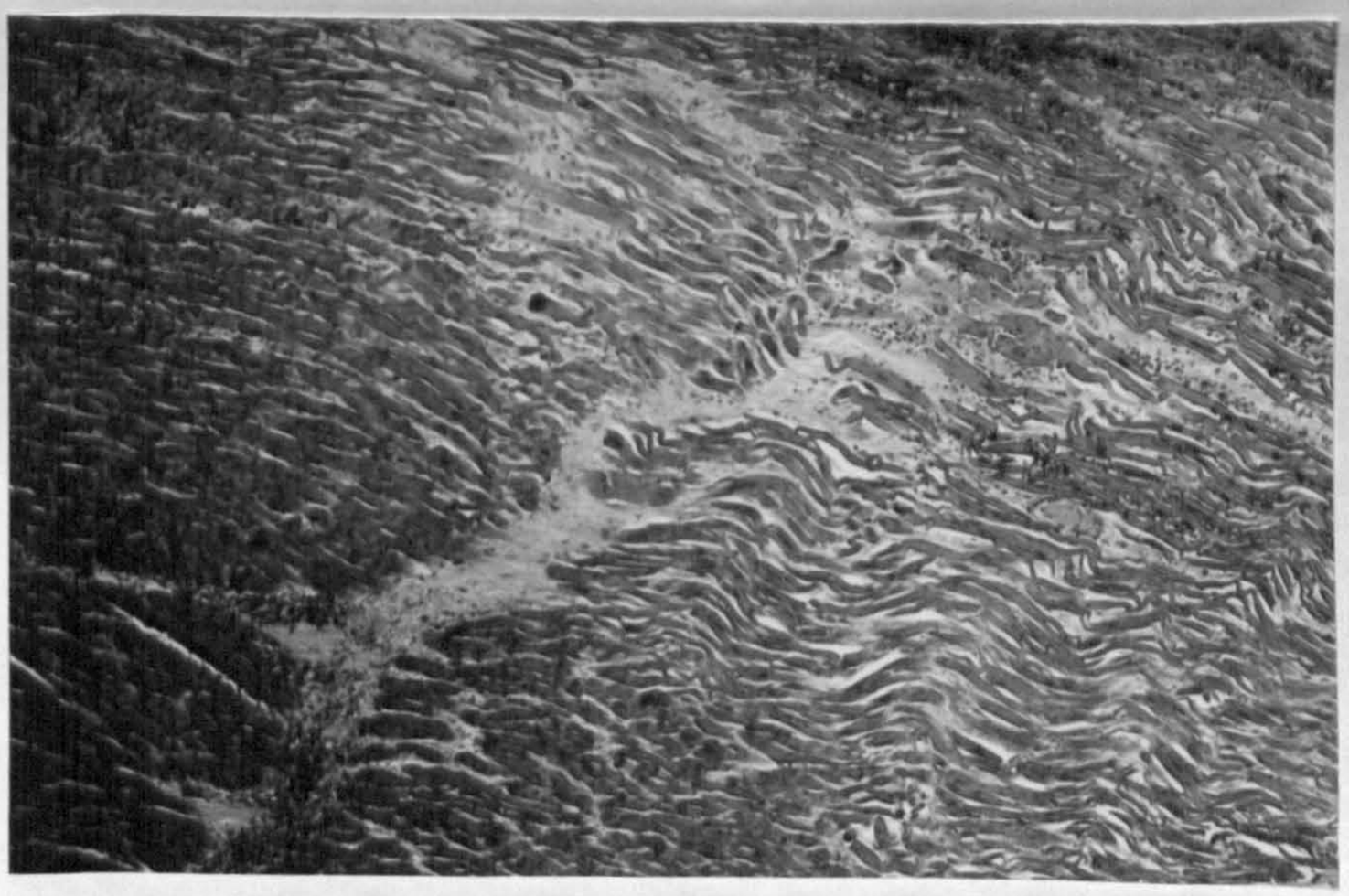


Figure 87: Wavy fibres at the edge of a myocardial infarct in a man of 56 years. Haematoxylin and eosin, X 125.

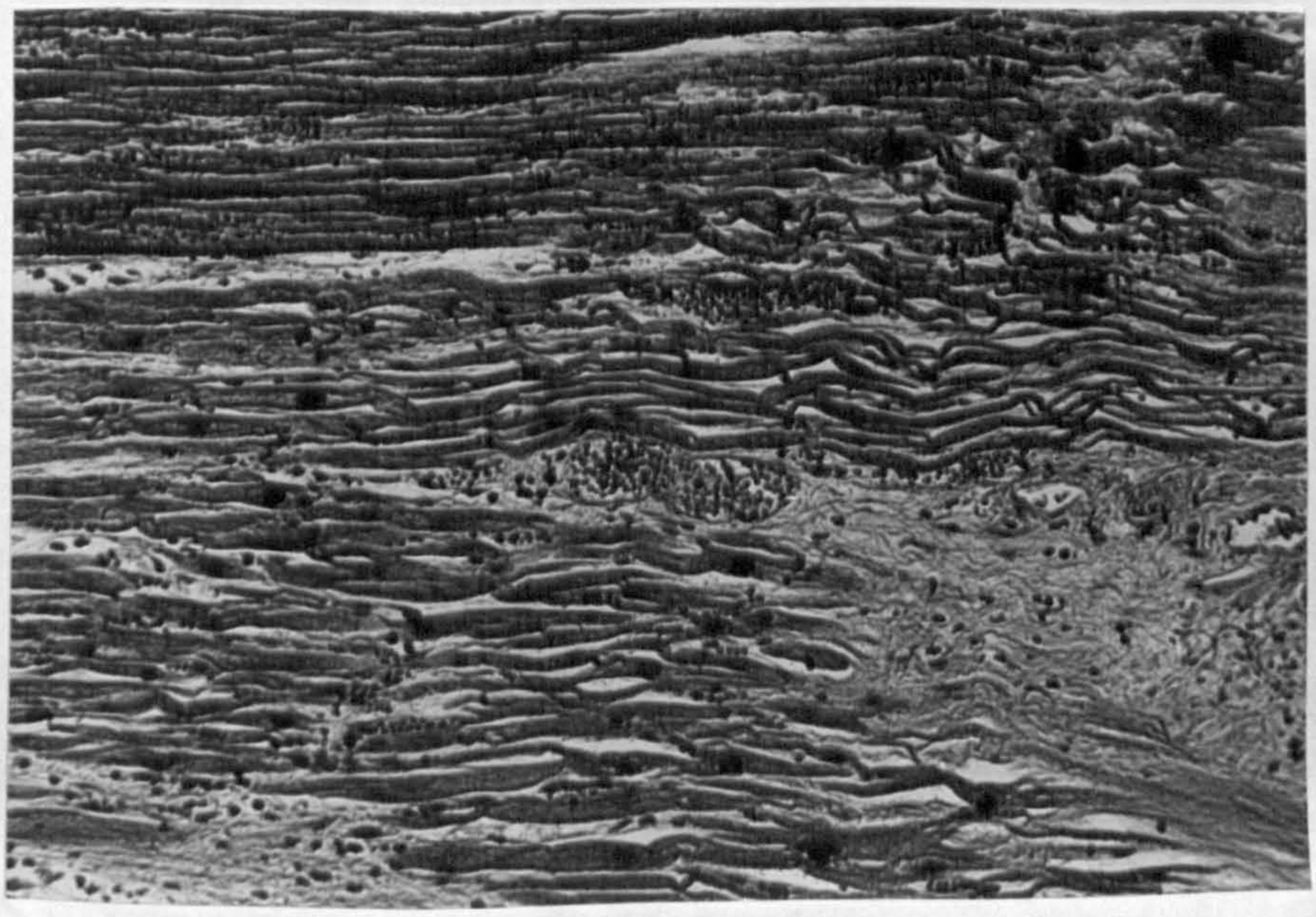


Figure 88: Wavy fibres at the edge of a myocardial infarct in a man of 32 years. Haematoxylin and eosin, X 125.

Figure 89: Wavy fibres infiltrated by polymorphnuclear leucocytes in myocardial infarction of a woman of 70 years.
Haematoxylin and eosin, X 125.

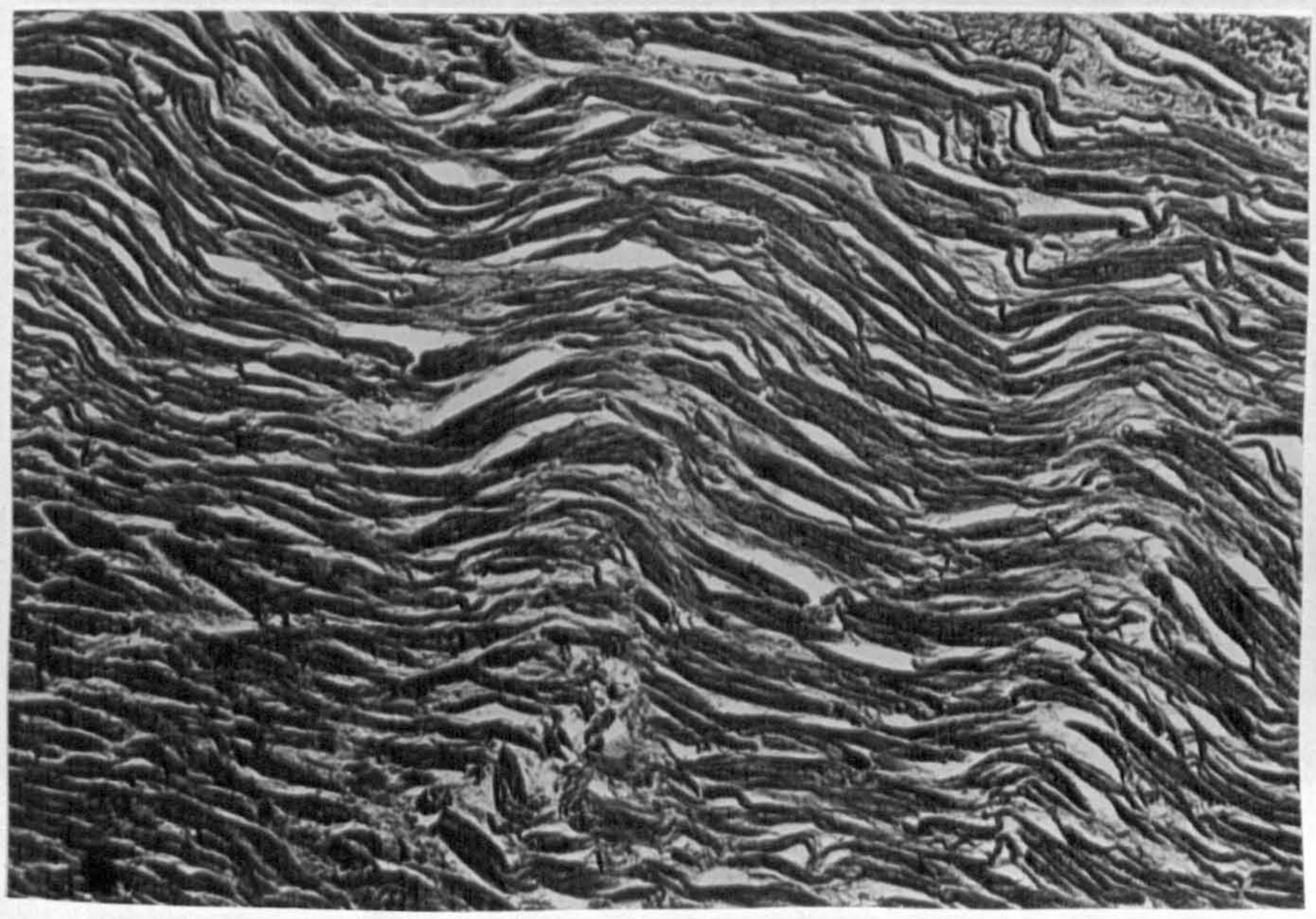


Figure 90; Wavy fibres in an autolysed normal heart of a man of 64 years.
Haematoxylin and eosin, X 125.

CHAPTER V

DISCUSSION

Studies on the morphologic features of acute myocardial infarction in man showed that there is a relatively long interval between the moment of damage to the myocardium and the appearance of the first micro- and macromorphologic changes. Light microscopic alterations do not permit any determination as to the time of cell damage before five or six hours, while unequivocal macroscopic changes require fifteen to forty-eight hours to become apparent (Mallory, White and Salcedo-Salgar, 1939; Lodge-Patch, 1951). Thus, the accurate diagnosis of acute myocardial infarction at autopsy frequently poses a problem to the pathologist when time has been too short for the development of detectable morphological changes in the myocardium. Many investigators were thus stimulated to search for early recognisable changes by various different methodologies. These include biochemical analysis (particularly of tissue electrolytes), ultra-structural studies, glycogen and lipid histochemistry, enzyme histochemistry, and histological staining and characteristics.

Selection of the appropriate method for the identification of early myocardial infarction in necropsy material

Electrolyte changes have been found in myocardial infarction by many workers, using chemical analysis of tissues or histochemical techniques. After producing coronary insufficiency in dogs, chemical analysis of the infarct showed about ten per cent loss of potassium during the first hour but, after this time, it rapidly disappears and,

at about twelve hours, the intracellular potassium is nearly equal to the concentration of this ion in the extracellular fluid. By histochemical methods, no change is observed until after six hours at which time a decrease of the potassium in the cell is observed, and by twelve to fifteen hours there is complete loss of potassium (Crout and Jennings, 1957; Jennings, Crout and Smetters, 1957; Jennings, Wartman and Smetters, 1957). Lowry, Gilligan and Hastings (1942), also working with dogs, noticed an increase in the sodium content accompanying potassium depletion. By cannulating the coronary sinus of dogs, Denis and Moore (1938) were able to measure a rise in the serum potassium level of blood samples removed from it seven minutes after ligation of a coronary artery. Klionsky (1960) observed definite evidence of potassium loss from cardiac cells twenty-one hours after coronary ligation in the rabbit.

Jennings, Sommers, Smyth, Flack and Linn (1960) reported an increase of sodium, chloride and water, and a decrease of potassium and phosphorus in a posterior papillary muscle infarct of dogs produced by ligation of the left circumflex coronary artery (Jennings, Wartman and Zudyk, 1957). It was further reported that the electrolyte changes occurred much more quickly in dogs exposed to transient ischaemia than in dogs with comparable periods of permanent ischaemia, and were well established after less than an hour's recirculation following forty minutes temporary occlusion. This is because diffusion of electrolytes into andout of infarcts caused by permanent occlusion is a slow process, as potassium ion has to leave the injured cells and then the ischaemic area, while sodium has to enter the ischaemic focus and then the injured cells. In contrast the injured cell in contact with the freely flowing blood supply in

transient ischaemia loses potassium and gains sodium quickly (Jennings, Sommers, Kaltenbach and West, 1964).

The ionic shifts in myocardial infarcts were explained as an increase in cell membrane permeability as an initial response to anoxia (Elsbach and Schwartz, 1959; Woodin, 1960; Whalen, Hamilton, Ganote and Jennings, 1974; Jennings, Ganote and Reimer, 1975; Willerson, Scales, Mukherjee, Platt, Templeton, Fink and Buja, 1977).

Zugibe, Bell, Conley and Standish (1966) described alterations in the sodium to potassium ratio as a very early alteration in the myocardium in infarction: they introduced a method for use at autopsy in the absence of gross and microscopic changes. The rationale of their K/Na method derives from the loss of K and gain in Na following the death of myocardial cells; the ionic ratio substantially changes within minutes after myocardial injury. The method described identifies an infarct by virtue of membrane changes affecting electrolyte transport.

By analysing the K/Na ratios in the supernatant of myocardial homogenates sampled from anticipated areas of infarction, Zugibe, Bell, Conley and Standish (1966) were able to identify infarcts at autopsy which were not demonstrable histologically. A low ionic ratio compared to the control samples obtained from the same heart is diagnostic for early infarction. McVie (1970) examined twenty hearts from cases of sudden death; they were found to be infarcted by the ionic (K/Na) ratio method, but not by histological examination.

Although the ionic ratio (K/Na) method appears simple to perform, it does not delineate an infarct in gross slices and its usefulness may be limited by possible sampling errors arising from the lack of topographic demonstration of infarction.

The relation between myocardial ischaemia and glycogen content of the heart is well known. The major part of the energy necessary for myocardial contraction is produced by the aerobic reactions of the citric acid (Krebs) cycle. The normal human heart utilizes fatty acid, glucose and amino acids (Bing, 1961; Green and Goldberger, 1961). However, under anoxic conditions, as in infarction, the main source of pyruvate in the ischaemic area derives from the anaerobic degradation of glycogen (Bing, 1961). Therefore, for the production of the same amount of energy much more carbohydrate must be utilized during anoxia, and this is in excess of thirty-fold. Consequently, it is logical to assume that the first histochemical indication of myocardial ischaemia would be the disappearance of glycogen from the infarcted area, a finding consistently demonstrated in experimental myocardial infarction five to ten minutes following ligature of the coronary artery (Yokoyama, Jennings, Clabaugh and Wartman, 1955; Kaufman, Gavan and Hill, 1959; Neoral, Kolin, Kodousek and Kvasnicka, 1959; Klionsky, 1960; Shnitka and Nachlas, 1963; Bajusz and Jasmin, 1964a; Sommers and Jennings, 1964; Fine, Morales and Scerpella, 1966; Kent, 1966; Krug and Korb, 1966; Seifert, 1967a; Mikami, 1968; Buss, 1970).

In human myocardial infarction, Wittels and Reiner (1960) found glycogen to be absent from the lesion regardless of the duration of the infarct, but glycogen was widely deposited throughout the viable

myocardium surrounding the fresh infarct. They stated that the distribution of glycogen in the viable portions of the left ventricle was related to the topography and age of the infarct. Glycogen was distributed widely outside the infarct but, upon healing, it became progressively limited to a narrow zone bordering the infarct. However, the accumulation of glycogen in the uninvolved area of infarcted human heart, as noted by Wittels and Reiner (1960), was not observed by Morales and Fine (1966).

Although the glycogen stores within myocardial fibres appear to be exhausted after five minutes' dependence on anaerobic metabolism, the use of glycogen histochemistry as an indicator of early myocardial infarction is not of particular use in studying autopsy cases. Mowry and Bangle (1951), and Wittels and Reiner (1966) reported that the small amount of glycogen and its irregular distribution in autopsy material invalidates its depletion as a reliable tool for detecting human myocardial infarction. Postmortem autolysis caused progressive loss of stainable glycogen in the canine myocardium; the sequence of changes were similar to that seen in ischaemic injury (Kent, 1957; Merrick and Meyer, 1954; Wittels, Reiner and Frank, 1959). Yokoyama, Jennings, Clabaugh and Wartman (1955) noted that the rate of glycogen depletion in the canine myocardium was similar in coronary artery ligation and postmortem autolysis. In addition, electron microscopic studies revealed significant changes due to postmortem autolysis which are almost identical to those seen in the ischaemic fibres (Herdson, Kaltenbach and Jennings, 1969).

It was observed in human myocardial infarction that, preceding the appearance of leucocytic infiltration or simultaneously with it, the necrotic glycogen-free muscle became PAS-positive. However, this reacting material was not digestible with diastase. Diastase-resistant PAS reactivity was observed in infarcted canine myocardium three to six hours after coronary artery ligation (Yokoyama, Jennings, Clabaugh and Wartman, 1955; Kaufman, Gavan and Hill, 1959; Fine, Morales and Scerpella, 1966; Kent, 1966; Krug and Korb, 1966; Seifert, 1967a).

The nature of the diastase-resistant PAS-positive material is not clear. French (1952) observed a similar reaction in degenerating myocardial fibres of potassium-deficient rats. Sommers and Jennings (1964) observed accumulation of a diastase-resistant PAS-positive polysaccharide in myocardial fibres within twenty minutes of recirculation following temporary coronary occlusion, and after four hours following permanent ligation. As the intensity of the PAS reaction increased with the length of recirculation, they concluded that the stained material diffuses into the necrotic fibres, and that it was possibly a plasma glycoprotein. In this connection, Kent (1966) correlated the appearance of the diastase-resistant PAS-positive material in the sarcoplasm of infarcted canine myocardium with the entry of plasma proteins into the dying myocardial fibre. He identified these plasma proteins as albumin, IgA (globulin) and fibrinogen by immunofluorescence histochemistry.

Although the diastase-resistant PAS-positive reaction is not influenced by postmortem autolysis, it does not seem to solve the problem

of identifying early myocardial infarction. Thus, its appearance coincides with the obvious histological criteria of myocardial infarction, such as leucocytic infiltration (Wittels and Reiner, 1960).

Lipid histochemical studies, using oil red O, showed neutral fat droplets in the sarcoplasm of ischaemic canine fibres, distributed at the poles of the sarcolemmal nuclei and between the myofibrils at thirty minutes to twelve hours after coronary artery ligation (Kent and Diseker, 1955; Kaufman, Gavan and Hill, 1959; Neoral, Kolin, Kodousek and Kvasnicka, 1959; Klionsky, 1960; Shnitka and Nachlas, 1963; Fine, Morales and Scerpella, 1966; Sakurai, 1977).

In human myocardial infarction, neutral fat appears five to twelve hours after coronary artery occlusion (Reiner, Wittels, Barrnett and Rutenberg, 1955; Morales and Fine, 1966). Mallory, White and Salcedo-Salgar (1939) observed fatty change in the necrotic fibres, especially at the edge of an infarct. They attributed the occurrence of the fat to the state of the myocardium previous to infarction and assumed that, if the infarcted myocardium previously had insufficient circulation, fatty degeneration would occur and this infarcted muscle would contain fat droplets, whereas if the muscle were entirely normal before infarction it would show only a small amount of fat. It has been suggested that some of the fat seen in ischaemic myocardial fibres results from unmasking of cellular lipids (Wartman, Jennings, Yokoyama and Claubaugh, 1956); these authors also suggested that the fat originated from circulating plasma lipids. This last suggestion was supported by the ultrastructural observation of enlarged lipid-containing vacuoles in the ischaemic rat myocardium, resulting from

accumulation of lipid metabolites in ischaemia (Page and Polimeni, 1977). Dixon (1958) proposed that fatty degeneration caused by hypoxia reflects diminished local synthesis of lipotropic agents (e.g. protein vehicles) for lipid transport. Furthermore, it can be proposed that hypoxia would decrease the local catabolic oxidation of triglycerides.

Hack and Ferrans (1960) identified a nitrogen-free plasmalogen in infarcted canine myocardial tissue, which first appeared as a few small sudanophilic droplets between some of the myofibrils. At twelve hours the sarcosomes were swollen and stained less intensely for plasmalogen, became more sudanophilic and finally disappeared, resulting in fine droplets of plasmalogen, which by twenty-four hours coalesced to become large fat droplets along the muscle fibres. This was interpreted by Adams (1967) as chemical degradation of hydrophilic phospholipid into sudanophilic lipid globules. However, it is difficult to be sure that the pseudoplasmal reaction (oxidation of ethylene bonds) had not contributed to this reaction.

The development of fatty change in the infarcted myocardium does not seem to be affected by postmortem autolysis. However, it is not particularly useful in diagnosing early myocardial infarction, since neutral fat first appears only six to twelve hours after coronary artery occlusion (Reiner, Wittels, Barrnett and Rutenberg, 1955; Morales and Fine, 1966), which coincides with the appearance of histological evidence of the lesion.

Electron microscopic appearances in infarcted myocardium have been reported by many investigators (Bryant, Thomas and O'Neal, 1958;

Caulfield and Klionsky, 1959; Herdson, Sommers and Jennings, 1965; Herdson, Kaltenbach and Jennings, 1969; De La Iglesia and Lumb, 1972; Kloner, Ganote, Whalen and Jennings, 1974; Jennings and Ganote, 1974, 1976; Buja, Dees, Harling and Willerson, 1976; Sakurai, 1977; Kloner, Fishbein, Hare and Maroko, 1979). The descriptive results obtained are almost the same in each report. Ultrastructural changes may begin to appear as early as five to fifteen minutes and are characterised by disappearance of perinuclear glycogen granules, nuclear alterations - such as clumping of nucleoplasm - and relaxation of myofibrils. Swelling of mitochondria appears in twenty to thirty minutes; the cristae become more distinct; electron dense granules appear in the mitochondria; and this is followed by mitochondrial rupture in four to five hours. At the same time, nuclear chromatin marginates, sarcotubules dilate and the sarcolemma stretches and breaks.

However, many of these changes, which were interpreted as due to ischaemia, were observed in autolysed myocardium (Herdson, Kaltenbach and Jennings, 1969; Sakurai, 1977). This invalidates electron microscopic examination in routine practice to detect very early infarcts; this is in addition to the disadvantage of being expensive and exceedingly time-consuming. Electron microscopy does, however, allow a better understanding of the sequential events in cellular ischaemia.

Calcium deposition in the myocardium has been described in both temporary and permanent myocardial infarcts (Lowry, Gilligan and Hastings, 1942; Yokoyama, Jennings, Wartman and Clabaugh, 1956). Calcium deposits can be demonstrated in occasional degenerating fibres with Alizarin red or

by microincineration (Sommers and Jennings, 1964). Calcification usually involves small clusters of cells running parallel to the border of the infarct, and is mainly related to mitochondria. In myocardial infarcts caused by temporary occlusion, Sommers and Jennings (1964) suggested that such calcium was brought in by recirculating blood or, as in the case of permanent occlusion, by diffusion from collateral vessels (Yokoyama, Jennings, Wartman and Clabaugh, 1956). However, calcium deposition does not seem to be a consistent finding in early myocardial infarction.

Enzyme histochemical diagnosis of myocardial infarction was a natural consequence of biochemical investigations and experimental studies that demonstrated that infarcted muscle is rapidly depleted of various enzymes, and that these appear in active form in the blood that drains from the infarcted myocardium (LaDue, Wróblewski and Karmen, 1954; Nydick, Wróblewski and LaDue, 1955; Wróblewski and LaDue, 1955; Wróblewski, Ruegsegger and LaDue, 1956; Hess, MacDonald, Frederick, Jones, Neely and Gross, 1964; Smith, 1967). The leak of myocardial enzymes into the circulation is attributed to disturbed integrity of cell membrane permeability in the injured myocardial fibre (Jennings, Kaltenbach and Smetters, 1957). Evidence about leakage of enzymes from the infarcted area into the circulation has been provided by some investigators (Ruegsegger, Nydick, Freiman and LaDue, 1959; Strandjord, Thomas and White, 1959; Kjekshus, 1976). The simultaneous elevation of enzyme levels in the blood is regarded as evidence of irreversible myocardial damage (Hamolsky and Kaplan, 1961).

As the heart muscle cells are characterised by the highest activity of oxidoreductive enzymes, Wachstein and Meisel (1955) examined the distribution of one of these enzymes, succinate dehydrogenase, in human myocardial infarction and in experimentally induced myocardial infarction in rats. Using frozen sections, they noticed reduced enzyme activity as early as one and a half to two hours after the onset of acute symptoms in many muscle fibres that showed no unequivocal microscopic evidence of damage with routine stains. Succinate dehydrogenase activity was also rapidly reduced in the experimentally infarcted rat myocardium. They recommended the use of the histochemical staining reaction of succinate dehydrogenase for the evaluation of myocardial damage under experimental conditions and in necropsy material.

Since then, many enzymatic histochemical studies have been concerned with identifying myocardial infarction. A decrease in cellular succinate dehydrogenase and other oxidoreductive enzyme activity in areas of myocardial ischaemic injury has been demonstrated and described by many investigators. Kent and Diseker (1955), Lushnikov (1963), Shnitka and Nachlas (1963), Bajusz and Jasmin (1964a) observed loss of succinate dehydrogenase activity from the infarcted myocardial fibres four to six hours after experimental coronary ligation in the dog, rat and rabbit. NADH and NADPH tetrazolium reductase activities were reduced at four to six hours in experimental myocardial infarction in the dog (Lushnikov, 1963). Cytochrome oxidase activity was noted to decline at ninety minutes in experimental infarction in the rabbit (Mikami, 1968), in contrast to six hours in the dog (Shnitka and Nachlas, 1963), and within

thirty-six hours after coronary occlusion in man (Burstone and Miller, 1961). The activity of lactate dehydrogenase was observed to be sometimes maintained for a while in the ischaemic area in experimental infarcts in dogs indicating the temporary persistence of anaerobic glycolysis (Lushnikov, 1963; Cox, McLaughlin, Flowers and Horan, 1968). Aerobic respiratory enzyme activity was observed to be lost earlier from the sarcosomes of myocardial fibres than from the A bands, which at first may exhibit apparently increased activity (Shnitka and Nachlas, 1963). Fine, Morales and Scerpella (1966) reported that B-hydroxybutyrate and iso-citrate dehydrogenases are the first enzymes to decline in activity, at one and a half to four hours, in experimental infarction in the rat. The loss of B-hydroxybutyrate dehydrogenase and glutaminase I were reported to be the most sensitive indicators of early human myocardial infarction, the activity of which is lost one and a half hours after coronary occlusion (Morales and Fine, 1966).

Sakurai (1977) observed loss of phosphorylase activity from myocardial fibres ninety minutes after experimental ligation of the coronary artery in the rabbit, while a loss of phosphorylase activity was observed as early as a few minutes from the experimentally induced lesion in the rat myocardium (Bajusz and Jasmin, 1964a and b; Fine, Morales and Scerpella, 1966). Monoamine oxidase activity was also observed to disappear early and, likewise, was reported to be a sensitive indicator of early myocardial infarction.

It seems to be generally accepted that enzymatic histochemistry is of value in the histochemical diagnosis of early myocardial infarcts, for such changes are clearly apparent before structural alterations

can be convincingly demonstrated with conventional staining methods. However, if the infarct cannot be identified and localised with the naked eye at autopsy, one or two random blocks might well fail to detect its presence. It was for this reason and because recognisable morphological alterations are late to appear in myocardial infarction that macroscopic enzyme histochemistry was applied to the problem.

Neoral, Kolin, Kodousek and Kvasnicka (1956, 1959) introduced their original dehydrogenase macroreaction with tellurite for the identification of early myocardial infarction in the gross. The principle of the reaction is an enzymatic reduction of potassium or sodium tellurite (an oxidation-reduction indicator) to black metallic tellurium mostly by the action of malate dehydrogenase. Normal heart muscle is rich in malate dehydrogenase and, accordingly, stains black. In contrast, infarcted muscle depleted of enzyme does not appreciably change colour. The results of their studies in experimental myocardial infarction in dogs revealed ischaemic areas in the transversely cut heart slice as early as five hours after coronary artery ligation.

Using the tellurite method with transversely cut heart slices at autopsy, Kolin, Neoral and Kodousek (1959) were able to identify the lesion in a patient who died seven hours after the onset of the acute symptoms, while Kolin and Neoral (1960) identified recent ischaemic changes as early as five hours after coronary artery occlusion.

Because of the three to eight hours incubation time required by the tellurite method, Sandritter and Jestädt (1957/1958) tried the

triphenyl tetrazolium reaction (TTC - reaction). This is based on the same principle as the tellurite method but involves a different hydrogen acceptor - triphenyl tetrazolium chloride - which stains normal heart muscle brick-red. The TTC-reaction requires only fifteen to forty minutes incubation. The earliest infarct they recognised was of three to four hours duration after the onset of clinical symptoms, in contrast to the five hours' duration infarct recognised by Knight (1967). In experimentally induced myocardial infarction in dogs, the TTC-reaction revealed an infarct as early as two hours after coronary artery ligation (Lie, Pairolero, Holley and Titus, 1975).

As the brick-red colour-product of the TTC-reaction deposited in the normal heart muscle of the transversely cut heart slice did not provide a good colour contrast with the faintly stained or unstained myocardial lesion, Nachlas and Shnitka (1963) modified the dehydrogenase macroreaction by using a different hydrogen acceptor, nitroblue tetrazolium (NBT) as an oxidoreduction indicator. The dark blue formazan pigment deposited in normal heart muscle clearly provided a better colour contrast than the faintly stained or unstained infarcted myocardial fibres. The results of their experimental studies, in dogs, using the NBT method, demonstrated the area of infarction as early as two hours after coronary artery ligation. Their subsequent experience with NBT in slices of twenty-three hearts obtained at autopsy from patients with a clinical history of ischaemic heart disease revealed an infarct as early as eight hours after the onset of clinical symptoms.

The NBT dehydrogenase macroreaction, using the incubating medium described by Nachlas and Shnitka (1963), which consists of buffered stock nitroblue tetrazolium solution with added exogenous substrate, sodium succinate, has since been adopted by most investigators concerned with the identification of early myocardial infarction in the gross. However, the published reports on the application of the nitroblue tetrazolium method do not expand on how soon after infarction the NBT test is diagnostic in man. McVie (1970), who examined twenty cases of sudden death, suggested an interval of three and a half hours, while Ramkissoon (1966), who surveyed thirty-one hearts obtained from patients with a clinical history of arteriosclerotic heart disease, suggested an interval of eight hours. The NBT test was positive when there was no apparent gross change in seven out of the twenty-six cases examined by Kalderon (1968), and in fifteen cases out of the one hundred consecutive autopsies carried out by Anderson and Hansen (1973). The latter authors also suggested an interval of eight hours. Brody, Belding, Belding and Feldman (1967) observed an interval of eighteen hours when examining the hearts of thirty-one patients who died with suspected or proven myocardial infarction.

Evidence from experimental studies may not necessarily be applicable to man. In particular, metabolic rates are different among various species, and the rate of ischaemic membrane disintegration may accordingly differ. Thus, extrapolation of results from animal to man may be misleading.

The macroscopic application of enzyme histochemistry

The results obtained in the present study in general confirm the applicability of the dehydrogenase macroreaction with nitroblue tetrazollum test for the gross identification of early myocardial infarction. Enzymes tested in this study included cytochrome oxidase, monoamine oxidase, myoglobin peroxidase, phosphorylase, glutamic-oxaloacetic transaminase, creatine phosphokinase, leucine aminopeptidase, non-specific esterase and acid phosphatase. It was observed that the dehydrogenase and also the diaphorase macroreactions using nitroblue tetrazollum (NBT) gave the most reliable and consistent results for the gross identification of early myocardial infarction. However, the previously-used NBT incubation medium was modified. Various exogenous substrates were used with the dehydrogenase macroreaction. These included DL-B-hydroxybutyric acid, sodium salt; DL-lactic acid, sodium salt; sodium succinate; L-malic acid; DL-isocitric acid, trisodium salt; D-glucose-6-phosphate, disodium salt. It was found, however, that the non-specific dehydrogenase incubating medium using the heart's own endogenous substrate with added NAD (nicotinamide adenine dinucleotide; coenzyme I) was the most sensitive in detecting areas of recent myocardial damage and gave optimal results for the early recognition of myocardial infarction. The incubation medium for NADH tetrazollum reductase was equally valuable. The inclusion of the respiratory chain inhibitor, cyanide, in the incubating medium to direct electrons away from the cytochrome oxidase system towards the tetrazollum salt increased the final colour product and increased the consistency of results.

Using the NBT incubating medium as described above, it was possible to identify areas of recent myocardial damage in seventeen out of forty-eight cases of clinical age under one hour. In these seventeen cases there was neither detectable or suspected gross alterations nor microscopic evidence of the lesion. Fourteen of these were forensic cases. It is appreciated here, however, that the clinical age might be shorter than the pathological age and, indeed, this was true in four cases of estimated clinical age under one hour where the lesion was recognised at autopsy to be older and was confirmed histologically to be so. Nevertheless, even in these cases, the enzymatic macroreaction revealed the real extent of the infarct by delineating its boundaries, and was therefore a useful adjunct. The increase in contrast between viable and necrotic myocardium afforded by the stain made delineation of the infarct much more precise. In one heart pathological interpretation remained the same before and after use of the enzymatic macroreaction, while in the remaining three hearts the extent of the infarct was larger than had been seen at autopsy. In six other cases, where infarct was only revealed by the NBT test, subsequent histologic examination of the mirror-image surface revealed early histologic changes. The topographical distribution of the lesion in all the twenty-seven (17 + 4 + 6) NBT-positive hearts in this group (clinical 'age' up to one hour) demonstrated ten transmutal, fourteen zonal, one laminar, one isolated papillo-muscular and one mixed infarct.

Among the twenty-eight subjects suspected of harbouring a recent myocardial infarct of estimated clinical age one to five hours, the enzymatic macroreaction revealed the lesion in twenty-two hearts.

Histologic evidence of the lesion was absent in fourteen hearts, twelve of which were forensic cases. In a further four hearts, myocardial damage was suspected at autopsy and was confirmed by the NBT test which showed the lesion in the four hearts to be more extensive than had been suspected. In the twenty-six NBT-positive cases, the lesion was shown to be transmural in thirteen hearts, zonal in eight, laminar in two, and mixed in three hearts.

In the five to twelve hour clinical age bracket, the number of hearts with an unrecognized infarct at autopsy, which was revealed by the NBT enzymatic macroreaction, was thirteen out of twenty-one. Eight of these were forensic postmortems. Histologic evidence of the lesion was absent in three cases. A further seven hearts showed naked eye evidence of the lesion at autopsy, which was better defined by the enzymatic macroreaction. In five of these, the infarct was found to be more extensive than had been suspected at macroscopic evaluation. In eleven hearts the infarct was delineated as transmural, zonal in four, laminar in one, mixed in two, and two were described as an isolated papillo-muscular infarction.

The eleven hearts obtained from suspected cases of estimated clinical age twelve to twenty-four hours were all positive with the NBT test. Five of these were identified at autopsy, in two of which the findings before and after the enzymatic macroreaction were in accord, while in the remaining three hearts, the extension of the infarct was larger than observed at autopsy. In six hearts the infarction was transmural, zonal in four, and mixed in one heart. The four forensic cases included

in this group were all positive with the NBT test.

With the five hospital cases with ischaemic heart disease in this group, where the diagnosis had been established on laboratory (two cases) and electrocardiographic (three cases) grounds, the lesion was not identified in the gross - except after the NBT test - in three cases (two with laboratory evidence and one with ECG changes). Ordinary macroscopic examination gave rise to suspicion in a second case with ECG evidence of infarction, and this was confirmed by the NBT test. In the last case with ECG changes, the lesion was identified at autopsy and the pathological interpretation remained the same before and after the enzymatic macroreaction.

Although the clinical age was probably wrong and underestimated the age of the infarct in a proportion of cases, yet it can be stated that the NBT test, using the incubation medium modified in this study, was positive in the absence of gross and histologic evidence in 35.4% of cases under one hour and in 63.6% of cases between one to five hours. These findings contrast with others reported in the literature using the NBT test, where it is suggested that it becomes positive after an interval of eight to eighteen hours after the onset of clinical symptoms (Nachlas and Shnitka, 1963; Ramkissoon, 1966; Brody, Belding, Belding and Feldman, 1967; Anderson and Hansen, 1973), but the present results are somewhat in agreement with the three and a half hour interval suggested by McVie (1970).

False negative results were not observed. Out of the one hundred and eight suspected cases of harbouring a recent myocardial infarct, the enzymatic macroreaction did not reveal an infarct in twenty-four

hearts. In none of these was a lesion suspected at autopsy nor could it be confirmed histologically. In these NBT-negative cases it is possible that some other myocardial damage was responsible for sudden death. It has been reported that in sudden unexpected death, which is defined as death occurring instantaneously or within some minutes from the onset of the acute attack (Baroldi, 1965; Friedman, Manwaring, Rosenman, Donlon, Ortego and Grube, 1973; Davies and Anderson, 1975; Schwartz and Gerrity, 1975; Davies and Popple,1979), ischaemic heart disease is not necessarily the underlying cause. Even the presence of a fresh thrombotic occlusion is not a proof of subsequent infarct (Corday, Spritzler and Prinzmetal, 1949; Baroldi, 1965; Schwartz and Gerrity, 1975; Bashe, Baba, Keller, Geer and Anthony, 1975). Although the incidence of occlusive thrombosis in the NBT-negative cases of clinical age under one hour is high, the coronary blood vessels showed moderate to advanced atherosclerosis. Crawford, Dexter and Teare (1961), and Perper, Kuller and Cooper (1975) reported that significant atherosclerosis of the coronary arteries, with or without thrombosis, is a main pathological cause in sudden cardiac death. Bashe, Baba, Keller, Geer and Anthony (1975) suggested that sudden cardiac death appears to be an electrophysiological phenomenon occurring in chronically damaged hearts with advanced coronary atherosclerosis, and there appears to be a peculiar instability of the heart which is not necessarily enough to cause infarction but may be adequate to alter the electro-physiological properties of cell membrane, thus producing ventricular fibrillation. This might have been the case in the NBT-negative cases

of sudden death. However, this explanation is quite speculative.

In two further cases the macroenzymatic test was negative in the presence of equivocal gross alterations. However, the suspected naked eye appearance of the lesion did not correspond to the anticipated area of myocardium supplied by the occluded coronary artery. The suspected area of congestion in the myocardium in one case might well have been traumatic as a result of external cardiac massage used in an attempt to resuscitate the patient. In the second case, there was a slight possibility of ectopic infarction, which is an infarction resulting from occlusion of an artery not normally supplying the area but doing so through collaterals. Equivocal pallor was seen in the anterior wall of the left ventricle in this case, but fresh thrombotic occlusion was found in the proximal part of the left circumflex coronary artery and only mild atheromatous changes were seen in both the left anterior descending and right coronary arteries. Histological examination of these two cases did not disclose any microscopic changes.

It can be stated that the macroenzymatic changes in all the eighty-four NBT-positive cases were consistent with the ischaemic changes to be expected from occlusion of the particular coronary artery concerned. None of the fourteen hearts with myocardial infarction used as positive controls, where the lesion was identified at autopsy and confirmed histologically, showed a false negative macroenzymatic test.

In all cases where an infarct was identified or suspected at autopsy, it was confirmed by the enzymatic macroreaction. This contrasts

with the finding of Anderson and Hansen (1973), where a false negative result was obtained in one case. The lesion was clearly identified at autopsy as involving the postero-septal wall of the left ventricle and the posterior papillary muscle; this was confirmed by the NBT test in the postero-septal wall area but not in the posterior papillary muscle. They also observed persistent dehydrogenase activity in the centre of large fresh infarcts in other cases. They suggested that the local persistence of dehydrogenase activity results from lack of drainage from these areas. This, however, could be caused by reduced sensitivity of the NBT dehydrogenase macroreaction. The incubation medium they used contained an exogenous substrate, sodium succinate; this could have made the conditions for enzymatic reaction more favourable and, thus, masked areas of recent myocardial damage.

In a group of four patients who were expected to develop terminal myocardial ischaemia, Ramkissoon (1966) observed that a negative enzymatic reaction in the supposed lesion was diagnostic when the heart's own (endogenous) substrate was used. However, the distinction disappeared after the addition of substrate (sodium succinate). This was explained as an artefact due to the disappearance of endogenous substrate from normal heart muscle as a result of postmortem autolysis, and when the exogenous substrate was added the reaction returned in normal areas (Anderson and Hansen, 1973). This observation was originally made by Nachlas and Shnitka (1963), who reported on loss of endogenous substrate from the normal myocardium if the death-necropsy interval exceeded six hours, and recommended the addition of exogenous substrate, sodium succinate, to the NBT incubating medium to compensate for the endogenous

substrate loss. This idea was later supported by other workers (Ramkissoon, 1966; Kalderon, 1968; McVie, 1970; Anderson and Hansen, 1973). The findings in the present study, using NBT endogenous incubating medium with added coenzyme and cyanide, indicate that the dehydrogenase macroreaction with endogenous substrate is independent of the death-necropsy interval. Satisfactory macrostaining of the heart was obtained with a death-necropsy interval as long as one hundred and twenty-four hours, with storage mainly at 4^{o}.

False positive results were not observed under the conditions of this study, and none of the thirty-seven hearts used as negative controls showed an enzymatic macroreaction arousing suspicion of a false positive result. Jestädt and Sandritter (1959) found false positive results in five of their negative controls. They supposed that this was mainly caused by technical problems. They also observed false positive results in fatty degeneration of the heart (non-specific absorption of the formazan onto fat?) Nachlas and Shnitka (1963) reported that a weak enzymatic reaction is sometimes seen normally in the bulbospiral muscle of the left ventricle, the fibres of which run parallel to the surface of transverse heart slices (Wartman and Souders, 1950). They suggested that this may lead to less exposure of the sarcosomes to the reagent solution than when the muscle fibres are cut at right angles and that this could give a false positive result (reduced staining). This problem was not encountered in the present study, possibly because the inclusion of the respiratory chain inhibitor, cyanide, in the incubating medium to direct electrons away from the cytochrome oxidase system towards the tetrazole increased the intensity

of the final colour product, and increased the consistency of the results.

Using frozen sections, Rosa and Velardo (1954) introduced cyanide in dehydrogenase histochemistry to prevent dehydrogenation by the natural pathway of the cytochrome system. With the use of faster-reacting tetrazoles, such as nitroblue tetrazolium, the use of cyanide was reported to be unnecessary (Nachlas, Tsou, de Souza, Cheng and Seligman, 1957). Adams (1967) modified the nitroblue tetrazolium method described by Nachlas and Shnitka (1963) for the gross identification of myocardial infarction, by including cyanide in the incubating medium which also contained the exogenous substrate sodium succinate. The findings observed in this study support an effective role for cyanide in the enzymatic macroreaction.

In the eighty-four NBT-positive cases of myocardial infarction, the lesion was found in the left ventricle and, also in the right ventricle in thirty-two of these hearts. In sixty-four, the lesion was not recognised in the left ventricle until after the NBT test had been performed, and twenty-six of these included the right ventricular wall. Anderson and Hansen (1973) found fresh myocardial infarction in the left ventricle in forty of their one hundred consecutive autopsies, and in three of these also in the right ventricle. In fifteen of their cases, the infarct was revealed by the NBT test in the left ventricle, but it is not clear whether the right ventricle infarcts were seen before or after the enzymatic test. The negative enzymatic reaction (absent staining) in the three cases of isolated papillary

muscle infarction, observed in this study, has shown that these infarcts were clearly of recent origin. However, it is possible that these minor infarcts may have commenced well before they became clinically apparent.

Knight (1967) described the dehydrogenase macroreaction as a useful procedure for the forensic pathologist to screen inapparent infarction. Kalderon (1968) found the enzymatic macroreaction most useful in cases of sudden death. Out of twenty-six cases examined by the last author, seven revealed positive diagnostic results, five of which were cases of sudden death. The results obtained here confirm the applicability of the enzymatic macroreaction to the field of forensic pathology, and support the use of the method in cases of sudden death.

Davies and Robertson (1975) defined sudden death as death occurring within twenty-four hours following the onset of acute symptoms. This definition is reported to be widely used in epidemiological surveys (Paul and Schatz, 1971). Sudden unexpected death is also defined as death occurring instantaneously or within an estimated twenty-four hours of the onset of the acute symptoms (Davies and Anderson, 1975). The suspected cases of recent myocardial infarction included here were all estimated clinically of under twenty-four hours. So by definition these can be considered as cases of sudden death. The circumstantial evidence in cases of sudden unexpected death may be grossly misleading and the clinical history may be inaccurate in some cases and, indeed, this was so in twenty cases (18.5%) observed here

as adjudged by gross and histological appearances. Neveretheless, the enzymatic macroreaction revealed infarction long before it was possible by conventional autopsy or conventional histology, and it is apparent that the enzymatic macroreaction is of practical value in cases of sudden death, particularly in forensic pathology. Even though when the lesion could be recognised at autopsy, the increase in contrast between viable and necrotic myocardium afforded by the stain made the delineation of the infarct much more precise. Thus, it was possible to assess semi-quantitatively the proportion of the ventricle that had undergone infarction.

Perfusion of the intact heart with tetrazolium solution, followed by perfusion with formaldehyde solution, has been proposed for the postmortem delineation of the infarcted myocardium and to provide a stored preparation for long-term clinico-pathological correlative studies (Lichtig, Glacov, Feldman and Wissler, 1973; Feldman, Glacov, Wissler and Hughes, 1976). This technique, however, was criticised by Roesch, Kuch and Knieriem (1976), who observed extensive unstained areas after perfusion of the whole heart with tetrazolium solution. These areas proved to be artefacts, since they reacted positively after incubation of the heart slices.

Postmortem autolysis was reported to limit the applicability of the NBT enzymatic macrostaining. Nachlas and Shnitka (1963) found that postmortem autolysis prevented an adequate reaction with endogenous substrate after a six hour death-necropsy interval, and recommended the addition of exogenous substrate to compensate for the loss of the

endogenous one. They also found that macrostaining was satisfactory in hearts stored at 4^{o} for twenty-four hours, but became patchy after forty-eight hours, while in hearts stored at 25^{o} to 37^{o}, the method was invalid after eight hours. Kalderon (1968) limited the applicability of the method to cases with short postmortem interval, while McVie (1970) reported that the method is invalidated by postmortem autolysis but did not state the limiting time interval. Anderson and Hansen (1973, 1974) found the method to be applicable seventy-two hours after death, the bodies having been kept at 4^{o}. The present findings on the effect of postmortem autolysis on NBT macrostaining of the heart support these last authors, even though they did not use added coenzyme and cyanide. The macrostaining of the hearts which had been stored at room temperature between 18^{o} - 25^{o} for three days was not significantly diminished over this period. Although the intensity of the dark blue formazan pigment was slightly decreased on the third day, yet none of the heart slices showed patchy or erratic staining. The hearts, which were stored at 4^{o} and -17^{o} for two weeks, showed macroscopic staining reactions of the same intensity as at the beginning.

The present consistency of the macrostaining reaction, irrespective of the death necropsy interval and within the permissible period of autolysis, is attributable to the incubating medium used here. The inclusion of magnesium in the stock NBT solution (see Methods, page 88), appears to protect mitochondria. Magnesium ions probably exert their protective effect by binding ATP to the mitochondrion (Pearse, 1972), thus protecting this organelle against non-osmotic types of swelling when ATP is lost. The use of hypertonic non-

electrolytic media seems to be important in dehydrogenase histochemistry, and especially so in the case of soluble or partially soluble enzyme systems. The addition of polyvinylpyrrolidone (PVP) to the incubating medium as suggested by Novikoff (1956) did not prove particularly useful in the gross. Newer methods of improved enzymatic localisation in sections with substrate film and semipermeable membranes have been described by Meijer (1972, 1973) and by Lojda, Gossrau and Schiebler (1976). Meijer and Vloedman (1973) applied a semipermeable membrane to fresh frozen sections of tissue in order to limit diffusion of enzymes and, thereby, effect accurate localization. However, these new methods are only applicable at the microscopic level.

Heart muscle normally contains substrates, coenzymes and enzymes. Of these it would appear that the coenzyme is the first to be depleted. This view is supported by the observation that the limiting factor in the dehydrogenase macroreaction, as noted in this study, was the addition of coenzyme to the NBT incubating medium and not the addition of exogenous substrate. Equal maximal enzymatic macrostaining of the heart was obtained with NBT incubating medium containing either coenzyme NAD alone or exogenous substrate plus NAD. When heart tissue was incubated in NBT stock incubating solution alone, no macroreaction was observed. When coenzyme NAD was added to the incubating medium, a macroreaction was obtained. Thus, it was clear that coenzyme NAD was a limiting factor in the dehydrogenase macroreaction.

This observation on NAD loss from the normal myocardium in necropsy material, and the capacity of the NBT medium with added

coenzyme to reveal recent myocardial damage tempts one to suggest that there could be a leakage of coenzyme in early myocardial infarction from the ischaemic area into the circulation. If so, this might occur earlier than the leakage of enzyme.

Disturbed integrity of the cell membrane and its increased permeability in myocardial damage has been reported by many investigators (Bryant, Thomas and O'Neal, 1958; Caulfield and Klionsky, 1959; Herdson, Kaltenbach and Jennings, 1969; Jennings and Ganote, 1974, 1976; Buja, Dees, Harling and Willerson, 1976; Sakurai, 1977). Biochemical assays in myocardial infarction show a difference among various myocardial enzymes in the time interval before the particular enzyme begins to leak into the extracellular fluid. This was reported to depend on the molecular weight of the enzyme and the increasing damage to the cell membrane with time (Jennings, Kaltenbach and Smetters, 1957). Since the molecular weight of coenzyme is much smaller than that of enzymes (coenzyme I, NAD, molecular weight = 663.5; lactate dehydrogenase molecular weight = 140,000; Lehninger, 1975), earlier leakage of coenzyme from ischaemic fibres into the circulation would not altogether be unexpected. This could be of diagnostic and prognostic value in the chemical and clinical fields of myocardial infarction, but proof of this suggestion is beyond the bounds of this study. Biochemical assays of ischaemic fibres in experimental myocardial infarction in dogs by Govier, as early as 1945, showed disappearance of seventy to eighty-three per cent of NAD within two hours. This supports the above suggestion of coenzyme-leakage from the infarcted area into the circulation.

The conclusion that NAD is the limiting factor in the enzymatic macroreaction draws attention to the nature of the enzymatic macroreaction per se. The persistence of the so-called tetrazolium "dehydrogenase" reaction when NAD is added to the incubating medium leads one to consider that the reaction may in fact depend on NADH tetrazolium reductase within the tissue. This enzyme appears to be organelle-bound and does not seem to be readily solubilised as are specific dehydrogenase (with the exception perhaps of the succino-oxidase system). This speculation is supported by the observation that a strong reaction is obtained within the incubating medium (with added exogenous substrate) when phenazine methosulphate is added, indicating that the dehydrogenase enzyme had leaked into the medium. Furthermore, a strong "dehydrogenase" reaction is obtained with many systems where the enzyme is known to be soluble. This is most probably due to NADH (or NADPH) tetrazolium reductase in the tissue accepting electrons from dehydrogenation products within the incubating medium (Glenner, 1965; Adams, 1967). Presumably NAD accepts an electron during dehydrogenase activity in the incubating medium, so that it is reduced to NADH which is the substrate for NADH tetrazolium reductase in the tissue. Finally, it was observed that the addition of NADH gives identical results to those found when NAD was added; this further strengthens the above conclusion.

Farber and Bueding (1956) were the first to use phenazine methosulphate (PMS) in dehydrogenase histochemistry, and showed that the reduced PMS could, in turn, reduce tetrazolium salts quantitatively.

Since it reacted directly with reduced flavoproteins and reduced coenzymes, and then non-enzymatically with tetrazolium salt, PMS was able to speed the velocity of the tetrazolium reaction and completely to by-pass the endogenous diaphorase systems in the tissue. In this way, dehydrogenation can be accelerated and made independent of the diaphorase, and accurate localization of dehydrogenases was thus thought to be possible. Since then PMS has been widely used in dehydrogenase histochemistry (Conklin, Dewey and Kahn, 1962; Hitzeman, 1963; Brody and Engel, 1964; Fahimi and Amarasingham, 1964; Hashimoto, Kaluza and Burstone, 1964; Glenner, 1965; Hardonk, 1965; Conklin, 1966; Johnson, 1967). However, when the role of PMS was examined in this macroscopic study, artefactual non-selective staining of the heart slices was observed with endogenous substrate and was more prominent with exogenous substrate.

PMS has been reported to differentiate between "type I" and "type II" hydrogen pathways (Altman, 1972). This author reported that type I hydrogen represents hydrogen that is able to pass along the electron transport chain (diaphorase) and reduce NBT. Type II hydrogen is not able to reduce the tetrazole directly and is, therefore, only apparent in the presence of the intermediate electron acceptor - PMS. The effects of PMS were explicable in terms of known biochemical functions of tissues, where type I hydrogen is equated with metabolism, and type II hydrogen with biosynthesis. The present study does not lend itself to the concept of type I and type II hydrogen, and the suggestion that, in the presence of PMS, the tissue's own diaphorase systems can be by-passed, thus accelerating the dehydrogenase reaction,

and that the reduction of NBT can be more efficient was not successful at the gross level.

The incidence of coronary thrombosis in this study was high. In transmural infarction, fresh occlusive thrombosis was found in the related artery in 76.9% of cases; zonal infarcts showed an incidence of 80.6%; mixed infarction had an incidence of 85.7%; in isolated papillary muscle infarction, the incidence was 66.6%; whereas cases with purely subendocardial laminar infarction showed the lowest incidence (25%). All the fourteen cases used as positive controls showed occlusive thrombosis. Coronary atherosclerosis was found in nearly all cases and varied from moderate to severe. The findings on thrombosis agree with the findings of Horie, Sekiguchi and Hirosawa (1978), who reported a high incidence (80.3%) of thrombus formation corresponding to the site of infarction, contrary to others who reported on a low ratio of coronary thrombosis to acute myocardial infarction (Ehrlich and Shinohara, 1964; Baroldi, 1976).

It is beyond the bounds of this study to discuss the hypothesis that coronary thrombosis is secondary to acute myocardial infarction, as suggested by some workers (Branwood and Montgomery, 1956; Ehrlich and Shinohara, 1964; Roberts and Buja, 1972; Baroldi, Radice, Schmid and Leone, 1974; Baroldi, 1976). However, the findings obtained here of a high incidence of coronary thromboses in regional infarcts, and lower incidence in the subendocardial laminar variety is in accord with the findings and views of Davies, Woolf and Robertson (1976); Davies, Fulton and Robertson (1979) that regional myocardial infarction is

precipitated by fresh occlusive thrombosis, while stenosing triple vessel disease is generally responsible for the subendocardial laminar variety.

The presence of myocardial necrosis associated with absence of occlusive thrombosis and in the presence of no significant coronary artery disease, but apparently resulting from hypoxia and shock of varying pathogenesis was revealed by the enzymatic macroreaction in ten hearts from subjects who died of illness other than myocardial infarction. In none of these cases was acute myocardial infarction suspected clinically. One case was of chronic severe megaloblastic anaemia. It has long been known that anaemia has a profound effect on the heart. Opitz (1935) described areas of myocardial necrosis in patients suffering from severe anaemia. Buchner and von Lucadou (1934) demonstrated electrocardiographic changes and areas of myocardial necrosis in animals in whom severe anaemia had been produced. Anaemia complicating massive haemorrhage has also been reported to cause myocardial necrosis (Master and Jaffe, 1940; Kinney and Mallory, 1945). It is clear that the underlying mechanism of necrosis is the diminished oxygen-carrying power of the blood. The same may apply to the case of carcinoma of the stomach, as a result of the anaemia that is often associated with carcinoma of the stomach (Morson and Dawson, 1979).

There seems no doubt that the mechanism of myocardial necrosis revealed macroenzymatically in the case of subarachnoid haemorrhage was the state of shock resulting in hypotension and general failure of coronary perfusion (Ratliff, Hackel and Mikat, 1969; Delage,

Mullick and Irey, 1973). In addition, haemorrhage itself tends to produce tachycardia where the work of the heart is increased and the coronary blood flow is decreased owing to the shorter duration of diastole. Clinically evident intracranial haemorrhage has been correlated with electrocardiographic abnormalities which simulate myocardial ischaemia and infarction (Burch, Meyer and Abildskov, 1954; Cropp and Manning, 1960). Macroscopic evidence of myocardial damage in subarachnoid haemorrhage was demonstrated in this study macro-enzymatically, which agrees with histochemical findings in frozen sections of the heart in experimentally induced intracranial haemorrhage in mice (Burch, Sun, Colcolough, DePasquale and Sohal, 1967). These authors suggested that intracranial haemorrhage may trigger the release of large amounts of noradrenalin at the tissue level, and this in turn may produce segmental necrosis which follows the distribution of the adrenergic nerve endings in the myocardium. Their suggestion was based on the observation that the myocardial necrosis which followed experimentally-induced intracranial haemorrhage was focal and relatively normal uninvolved segments were interspersed between extensively damaged ones. This accords with the adrenergic nerve supply to the myocardium which is randomly distributed over individual myocardial fibres (Hirsch and Borghard-Erdle, 1961, 1962). This is supported by the observation that the myocardial lesion in haemorrhagic shock can be averted in the laboratory animal by B adrenergic blockade (Entman, Chang, Mikat, Martin and Hackel, 1965). Sympathomimetic amines, namely isoprenalin, have also been reported to induce myocardial necrosis in rats. However, the pathogenesis of isoprenalin-induced myocardial necrosis in the rat has been attributed to an increased

metabolic rate in the affected fibres and, hence, an oxygen demand which cannot be met by the coronary flow (Woolf, Davies, Shaw and Trickey, 1976; Collins and Bellings, 1976).

The myocardial necrosis revealed macroenzymatically in the other conditions could be generally classified as the poor coronary perfusion "syndrome". In calcific aortic stenosis, the coronary blood flow may have not been sufficient for the increased left ventricular work. In the case of rheumatic mitral stenosis, perhaps the associated increased pulmonary vascular resistance might result in low cardiac output, which together with the associated aortic valve stenosis may have led to poor coronary perfusion. In the case of acute corrosive poisoning, shock and hypotension could clearly cause poor coronary perfusion; the same applies to the case of chest infection in which the blood pressure was unrecordable six hours before death. In the case of coronary artery hypoplasia, the coronary arteries were not diseased but were of such small calibre as to cause inadequate coronary perfusion.

Although extensive fibrosis was seen at autopsy in a case of congestive cardiomyopathy, yet it was much better outlined after enzymatic macrostaining. In addition, staining revealed fibrosis of the right ventricle which was overlooked at autopsy. Electrocardiographic changes consistent with infarct pattern has been reported in congestive cardiomyopathy (Fowler and Gueron, 1965), the extensive fibrosis seen in this case might well represent healed infarction.

Congenital heart disease is frequently present in the Ellis-Van Creveld syndrome; atrial septal defect is the most common lesion, and

indeed this was present in this case. At autopsy the right ventricle was enlarged, in evidence presumably of a left to a right shunt. In time, the pressure in the right side of the heart would have increased until the shunt was reversed and cardiac failure ensued. This might explain the myocardial necrosis revealed macroenzymatically, which may have been due to poor coronary perfusion resulting from cardiac failure.

It appears that the underlying mechanism of myocardial damage in the above mentioned miscellaneous cases of myocardial necrosis was, in general, poor coronary perfusion. As the subendocardial fibres are the most vulnerable to ischaemic conditions, the anatomical pattern of myocardial necrosis is then expected to be of the subendocardial laminar variety (Levine and Ford, 1950; Estes, Entman, Dixon and Hackel, 1966; Davies, 1977). This was seen in the case of calcific aortic stenosis, rheumatic mitral stenosis and subarachnoid haemorrhage. In the other cases, although the necrosis was subendocardial, yet it was zonal in localization, except the case of severe megaloblastic anaemia, where the necrosis was nearly transmural. This is difficult to explain. Possibly this was due to difference in the vulnerability of the subendocardial fibres, or that the subendocardial plexus of vessels was efficient in one area, and not in another. Another possibility is abnormality of the intramyocardial vessels. Lastly, it has been reported that myocardial contraction impedes coronary flow (Sabiston and Gregg, 1957). The possible role of myocardial counter-pressure must also be considered in diminishing blood flow in some areas

of the myocardium and not in others; such irregular pressure might be brought about by ventricular dysrrhythmia in a terminal episode. It must be noted that, except in the case of congestive cardiomyopathy, histological examination of the mirror-image surface in these cases suggested that the necrosis was not older than five to twelve hours.

Although in these miscellaneous cases, it seems reasonable to attribute the positive macroreaction result (area of absent staining) to poor coronary perfusion, nevertheless it could not be denied that abnormal heart muscle (e.g. as in a cardiomyopathy) might in its own right show reduced or absent staining.

Myocardial necrosis complicating cardiac surgery has been observed in frozen myocardial sections by some workers (Morales, Fine and Taber, 1967; Hensen, Najafi, Callaghan, Coogan, Julian and Eisenstein, 1969). This complication of cardiac surgery was revealed macroenzymatically in two hearts included in this study, and may represent the end result of inadequate perfusion of the myocardium during the operation. Such a lesion was also demonstrated macroenzymatically in a third heart obtained from a patient who was undergoing surgical treatment of a ruptured aneurysm of the abdominal aorta, and this may have been the result of an overall fall in coronary perfusion. Myocardial necrosis seems to be a serious anatomical and probably functional complication in surgery and may significantly contribute to postoperative mortality.

Coronary air embolism, complicating the course of transfusion, was demonstrated by the enzymatic macroreaction to have caused myocardial

infarction. The occurrence of such infarction emphasizes an occasional risk in transfusion.

The abnormal diffuse dilatation of a coronary artery or ectasia (Aschoff, 1924; Markis, Joffe, Cohn, Feen, Herman and Gorlin, 1976) was encountered in one heart of a female patient in whom the macro-enzymatic test revealed recent zonal myocardial damage of the posterior wall of the left ventricle in the presence of an ectatic right coronary artery. This seems to fit well with the suggestion that coronary artery ectasia commonly results in occlusive coronary disease (Swanton, Thomas, Coltart, Jenkins, Webb-Peploe and Williams, 1978) as, due to poor perfusion pressure, such vessels are particularly prone to undergo thrombosis (Wilson, Adams and Brander, 1978). Swanton, Thomas, Coltart, Jenkins, Webb-Peploe and Williams (1978) found an incidence of 1.1 per cent of coronary artery ectasia (all men) in a series of one thousand coronary arteriograms. Measurements of coronary sinus flow in two of these patients showed flow in the range of patients with non-ectatic coronary arteries and, at cardiac surgery, flows down the grafts to ectatic arteries were in the same range as in grafts to non-ectatic vessels. They postulated that with a vessel of greater diameter but an equal or reduced flow the velocity of blood must be reduced, and they came to the conclusion that patients with coronary artery ectasia should be anticoagulated to prevent subsequent thrombosis (see above).

Age and ageing did not seem to have a direct effect on the macro-enzymatic reaction. At the other extreme, myocardial infarction as revealed macroenzymatically was seen in a number of subjects between the

second and fourth decade of life. The age of these subjects was twenty-six, thirty-two, forty-two, forty-four and forty-eight years. They were all men, and the overall incidence was 5.9%. Apart from coronary thrombosis, many factors seem to be intercorrelated such as smoking, obesity, hypertension, psychological stress, alcohol ingestion, high serum cholesterol values and oral contraceptives in women (Myers and Dewar, 1975; Salvador, Bizouati and Marco, 1976; Engel, 1976; Engel, Hundeshagen and Lichtlen, 1977; Bergstrand, Vedin, Wilhelmsson, Wallin, Wedel and Wilhelmsen, 1978).

Cytochrome oxidase was applied as a macroreaction in this study, but it did not prove to be a sensitive indicator of early myocardial infarction. The G-Nadi cytochrome oxidase reaction was so intense that a recent myocardial infarction, which was revealed in a heart slice stained with NBT endogenous medium, was not apparent in an opposing heart slice examined for cytochrome oxidase activity. This macroscopic finding contrasts with the finding of Morales and Fine (1966) who found an early depletion of cytochromc oxidase in recent human myocardial infarction. It is suggested here that heart muscle is rich in cytochrome oxidase which is not affected in early myocardial infarction. Possibly this survival is related to tight binding within the mitochondrion.

An early loss of monoamine oxidase (MAO) activity has been reported by Bajusz and Jasmin (1964a) in experimental myocardial infarction in rats. The activity of this enzyme was not investigated in human myocardial infarction. However, MAO macroactivity was found here not to be a sensitive indicator of recent myocardial damage. The earliest

loss of enzyme activity was observed in myocardial infarction of twenty-four to forty-eight hours duration.

Recent histochemical study on the activity of creatine phosphokinase (CPK), using a tetrazolium method, in experimental myocardial infarction in dogs, revealed an early macroscopic loss of enzyme activity, five hours after coronary artery ligation (Anderson, Popple, Parker, Sayer, Trickey, and Davies, 1979). By applying a tetrazolium method for the demonstration of CPK activity described by Sjövall (1967), which is essentially similar to the method used by these authors, it was observed that the final pathway in the enzymatic reaction for demonstrating CPK activity is by the way of the relevant tetrazolium reductase. This was noticed from the equations describing the reactions for the demonstration of CPK activity (see Methods, pages 109,110). This was confirmed when identical reactions were obtained when adjoining heart slices were incubated in CPK incubating medium with and without the specific substrate creatine phosphate. Indeed, a similar reaction was observed when a third heart slice from the same heart was incubated in a medium for the demonstration of NADPH tetrazolium reductase. Thus, it would appear that the histochemical demonstration of CPK activity is virtually the localization of NADPH tetrazolium reductase. When phenazine methosulphate (PMS) was added to the incubating medium to circumvent NADPH tetrazolium reductase and to bring about direct reduction of nitroblue tetrazolium salt, it resulted in non-selective staining of the heart slice, the same as observed when PMS was added to the incubating medium for dehydrogenases and diaphorases. The CPK

method appears to delineate infarctions in the gross, but the mechanism involved seems to be non-specific, and it is realised that it is not necessarily due to a specific loss of creatine phosphokinase.

Phosphorylase activity has been studied in human myocardial infarction by Morales and Fine (1966), but only at the microscopic level. Because of its major role in controlling glycogen metabolism, it was tried here as a test in the gross for early myocardial infarction. However, no macroenzymatic activity could be demonstrated. Morales and Fine (1966), observed a strong phosphorylase activity in the myocardium obtained from two open heart operations, but was absent in postmortem myocardium. Braunstein (1968), observed a rapid disappearance of phosphorylase activity from the myocardium after death. It would appear that phosphorylase is rapidly depleted by postmortem autolysis which explains why macroactivity was not obtained here. Thus, it cannot be used in diagnosing myocardial infarction at autopsy.

The latest contribution to the clinical diagnosis of myocardial infarction is the reported rise in the serum level of the respiratory pigment, myoglobin, in patients with acute myocardial infarction (Stone, Waterman, Harimoto, Murray, Wilson, Platt, Blomqvist and Willerson, 1977). However, the attempt made here at the histochemical demonstration of myoglobin in transverse heart slices, which is not reported before in the literature, was unsuccessful. Few isolated myocardial fibres were stained but no proper staining of the myocardium was seen. This could be either due to myoglobin degradation in

the postmortem myocardium, or due to its solubility as observed by some workers who investigated the histochemical localization of myoglobin in frozen sections of human and laboratory animals skeletal muscle (Drews and Engel, 1961; James, 1968; Morita, Cassens and Briskey, 1969).

A rise in the serum level of glutamic-oxaloacetic transaminase, GOT (or aspartate transaminase, AST), is associated with acute myocardial infarction (LaDue, Wróblewski and Karmen, 1954). The histochemical demonstration of the activity of this enzyme has not been investigated before, in human or experimental myocardial infarction. However, the attempt made here macroscopically to investigate its possible role in identifying acute myocardial infarction at necropsy was not successful. Non-homogenous and patchy staining of the myocardium was seen, making it difficult to assess the results precisely. Again, this could be attributed to postmortem autolysis. But as staining of some myocardial fibres was seen, the method is worth recommending for experimental studies in laboratory animals under more controlled conditions of postmortem autolysis.

Normal myocardial fibres contain no histochemically demonstrable aminopeptidase activity. The aminopeptidase that appears during myocardial infarction is at first located in the polymorphnuclear leucocytes which infiltrate the lesion about twelve to twenty-four hours after coronary occlusion. The macroscopic demonstration of aminopeptidase activity in human myocardial infarction has not been reported in the literature. It was attempted here in myocardial infarction of estimated clinical age twelve to twenty four hours,

to test whether the macroreaction can be diagnostic for myocardial damage at this time. However, the attempt was unsuccessful and no macroscopic enzyme activity could be demonstrated, presumably due to the small number of infiltrating cells at this time.

Acid phosphatase activity was demonstrated histochemically by Pearse (1964) in the perinuclear region of normal myocardium. Ultrastructural cytochemistry and subcellular fractionation of biopsies of experimentally infarcted dog myocardium showed that, two hours post infarction, the lysosomes were disrupted by ischaemia, resulting in intracellular release of acid phosphatase (Hoffstein, Weissmann and Fox, 1976). The histochemical demonstration of acid phosphatase was attempted here to test its possible use in identifying areas of myocardial damage. The macroenzymatic reaction revealed a homogeneous non-specific red staining of the myocardium. This could be due to the high solubility of the primary reaction product (PRP), α-naphthol (Barka and Anderson, 1962). This may lead to diffusion and non-specific staining when the PRP is simultaneously coupled with hexazotized pararosanilin to form the final reaction product (FRP).

Strong non-specific esterase microactivity in human myocardial infarction was observed by Morales and Fine (1966). Macroscopically, the histochemical demonstration has not been previously reported, and was attempted here to test its possible role in revealing early myocardial damage in the gross. However, the colour of the FRP, which is dark reddish brown, was so intense that contrast of colour between increased activity in myocardial infarction and normal myocardium was not

distinct, making it difficult to outline areas of myocardial damage.

On the basis of this study on the histochemical macroenzymatic reaction of the various enzymes tested for the early identification of myocardial infarction at necropsy, it is concluded that the nitroblue tetrazolium (NBT) method for dehydrogenases and diaphorases is the most successful method. On evaluating the conditions (see above) that provide maximum NBT enzymatic macroreaction as applied in this study, it is concluded that the non-specific dehydrogenase incubating medium, using the heart's own endogenous substrate, with added NAD (nicotinamide adenine dinucleotide; coenzyme I) and the respiratory chain inhibitor, cyanide, is the most sensitive in picking up areas of recent myocardial damage. The technique with this incubation medium is not in practice affected by postmortem autolysis, and within limits is independent of the death-necropsy interval. It was suggested above that coenzyme NAD is the first to be lost from the injured myocardium, possibly long before enzymatic depletion. This would be of clinical significance in the diagnosis and prognosis of acute myocardial infarction. The persistence of the tetrazolium "dehydrogenase" reaction when NAD is added to the incubating medium leads one to consider that the reaction mainly depends on NADH tetrazolium reductase (or diaphorase). The addition of phenazine methosulphate (PMS) to by-pass the endogenous tetrazolium reductases (or diaphorases) in the dehydrogenase macroreaction resulted in non-selective macrostaining of the heart slice, probably because PMS accelerated the transfer of electrons in solutions instead of in situ.

Histologic staining of the myocardium in early infarction

The non-histoenzymic phloxine-tartrazine stain, as described by Lendrum (1947), is introduced here as a microscopic aid in the identification of early myocardial infarction in paraffin sections. The method was successful in revealing early myocardial damage by selective staining of pre-necrotic myocardial fibres. It was observed that the affinity of the ischaemic fibres for phloxine was striking and dramatic long before myocardial fibres show any structural evidence of necrosis. The underlying chemical basis of the staining reaction is not clear, but since phloxine and tartrazine are both acidic dyes (Lillie, 1969), it is suggested that the underlying chemical mechanism in picking up ischaemic fibres is possibly the intracellular acidosis as a consequence of ischaemia, or much more likely is the exposure of amino groups. Postmortem autolysis did not appear to alter the staining results of normal or damaged myocardial fibres. The results of staining were consistent, and neither false positive nor false negative staining was observed. The method is regarded as reliable and reproducible.

The haematoxylin-basic fuchsin_picric acid (HBFP) stain (so-called fuchsinorrhagia) for the histochemical identification of myocardial ischaemia in tissue sections (Lie, Holley, Kampa and Titus, 1971) has been strongly recommended by some workers (Nayar and Olsen, 1974; Scherer and Masi, 1975; Degrel, 1976; Cooper, 1977; Rajs, 1979). However, when the method was applied here, inconsistent and unrellable results were obtained. The method depends particularly on the extent

to which the sections are differentiated. Hence, slight under-differentiation provided false positive staining and vice versa. The inconsistency found here accords with criticisms by other workers (Zugibe and Zugibe, 1973; Reepts, Borgers and Reneman, 1976; Woolf, Davies, Shaw and Trickey, 1976; Rose, Opie, Bricknell, 1976; Derias and Adams, 1978). Possibly a particular knack or skill is required to obtain consistent reliable differentiation.

The acid fuchsin stain (so-called fuchsinophilia) for the histochemical identification of early myocardial infarction in tissue sections (Poley, Fobes and Hall, 1964) does not seem to have gained as much support as HBFP. Lie (1968) regarded the method as reliable. Although the results observed here were more consistent than with HBFP, yet the method did not provide sharp differential staining between the normal and necrotic fibres. It was also observed not to be clearly positive until structural changes in myocardial fibres were seen microscopically. Thus it was considered unsuitable as an early test. This agrees with the findings of other workers (Morales and Fine, 1966; Zugibe, Bell, Conley and Standish, 1966; Zugibe and Zugibe, 1973; Woolf, Davies, Shaw and Trickey, 1976).

Non-specific morphologic alteration of myocardial fibres

It has been previously reported that the presence of wavy myocardial fibres is a characteristic and diagnostic feature of early myocardial infarction (Bouchardy and Majno, 1971/1972, 1974). Infarcted myocardial

fibres use up its energy-rich substrates so that it would be expected to take up a relaxed extended state. This would lead to an excess "slack" or length in some of the muscle fibres surrounding the infarcted zone, as such fibres would perforce become wavy. However, in recent studies on myocardial infarction, considerable suspicion has arisen about the specificity and reliability of the wavy fibre as a histopathological index of myocardial infarction (Rose, Opie and Bricknell, 1976; Sakurai, 1977; Derias and Adams, 1978). In the present study, the primary, secondary and the less frequent tertiary-order wavy fibres, as described by Bouchardy and Majno (1974), were seen with equal frequency in normal and infarcted human myocardium; the wavy fibres in the two conditions were indistinguishable. Waviness of myocardial fibres was also seen in the autolysed heart. Previous studies on the nature of the wavy myocardial fibre (Derias and Adams, 1979), showed that such fibres were present in normal rat myocardium. They were also present in sections of infarcted rat myocardium, which were kindly provided in 1977 by Professor Neville Woolf. They have also been observed by other workers in the infarcted rat myocardium (Ell, Langord, Pearce, Lui, Elliot, Woolf and Williams, 1978; Kloner, Fishbein, Hare and Maroko, 1979). Derias and Adams (1979) studied the effects of adenosine 5'-triphosphate (ATP) and 2,4-dinitrophenol (DNP) on the formation of the wavy fibre in the normal rat heart. It was thought that patchy absorption of ATP might promote patchy muscle contraction and intervening areas of wavy fibres. By contrast, uncoupling of oxidative phosphorylation by DNP might be expected to cause uniform relaxation and an absence of wavy fibres. The results

obtained were in favour of this assumption and have led to the conclusion that wavy fibres are caused by uneven contraction of myocardial fibres brought about by patchy preservation or loss of ATP, which may result from infarction or from postmortem autolysis. It must be emphasised that Bouchardy and Majno (1971/1972, 1974) carried out autopsies within twelve to a maximum of twenty-four hours postmortem, the bodies were maintained at 4^{o}, and their material did not include normal hearts.

The overall conclusion about wavy fibre is that it is useless as a histological index of infarction.

Summary and conclusions

With the object of identifying early myocardial infarction in the gross, various myocardial enzyme systems were examined in transversely cut slices of one hundred and seventy-nine human hearts obtained at necropsy. These included dehydrogenases, diaphorases, cytochrome oxidase, monoamine oxidase, myoglobin peroxidase, phosphorylase, glutamic-oxaloacetic transaminase, creatine phosphokinase, leucine aminopeptidase, non-specific esterase and acid phosphatase.

From this study, dehydrogenases and diaphorases using nitroblue tetrazolium as a redox indicator were found to be the most reliable methods. The conditions that provide the maximum nitroblue tetrazolium macroreaction involve the non-specific dehydrogenase incubating medium using the heart's own endogenous substrate with added NAD (coenzyme I) and added respiratory chain inhibitor, cyanide. This was the most sensitive medium for depicting areas of recent myocardial damage. The use of NAD and cyanide for the gross detection of the lesion is a new modification introduced in the present study.

The macroenzymatic reaction as described revealed areas of recent myocardial damage in eighty-four out of one hundred and eight hearts suspected of harbouring a myocardial infarct of estimated clinical age under twenty-four hours. The total number of hearts in which the lesion was not recognised or suspected at autopsy but was revealed by the nitroblue tetrazolium test was sixty-four. Twenty-three of these were cases of sudden unexpected death within one hour after clinical onset, seventeen of which showed no histologic evidence of the lesion; twenty

two cases were of estimated clinical age one to five hours, fourteen of which did not show histologic evidence; thirteen cases were of estimated clinical age of five to twelve hours, three of which showed absence of histologic evidence; six cases were of estimated clinical age of twelve to twenty-four hours, all of which were confirmed histologically. It is appreciated that the circumstantial evidence in cases of sudden unexpected death may be grossly misleading, and that the clinical age may be shorter than the pathological age, indeed this was probably true in twenty cases observed here, as adjudged by gross and histological appearances.

False positive results were not obtained, as far as can be ascertained within the limits of this study. In none of the thirty-seven hearts used as negative controls did the enzymatic macroreaction arouse suspicion of infarction. There was no false negative result. Out of the one hundred and eight suspected cases of recent myocardial infarction, the enzymatic macroreaction did not reveal an infarct in twenty-four hearts. In none of these cases was an infarct suspected at autopsy or confirmed histologically. In all fourteen hearts used as positive controls, the lesion was confirmed by the histochemical macroreaction. The twenty hearts of underestimated clinical age, in which the lesion was identified at autopsy, showed a positive enzymatic test.

Postmortem autolysis did not appear within limits to affect the enzymatic macroreaction. Within the permissible period of autolysis, macrostaining of the heart was not significantly diminished over seventy-

two hours at ambient temperature between 18° and 25°. With hearts stored at 4° and -17° for two weeks, the macrostaining reaction was of the same general intensity at the end as during the first twenty-four hours. The endogenous medium described here is essentially independent of the death-necropsy interval. Satisfactory dark blue staining of the heart, using the endogenous substrate, was obtained as long as one hundred and twenty hours after death, with storage mainly at 4°.

The subject's age and related ageing changes did not seem to have an effect on the macrostaining of the heart.

The macroenzymatic reaction revealed myocardial infarction of the left ventricular wall in sixty-four cases and in twenty-six of these the right ventricular wall was also involved. In all cases the left and right sided lesion was not suspected at autopsy.

The concurrence between the result of the enzymatic macroreaction and the histologic changes was convincing with regard to the histologic criteria followed in this study.

As reported previously in the literature, the incidence of occlusive thrombosis with regional infarcts was high (85.7%), and was low (25%) with the subendocardial laminar variety.

Myocardial necrosis following hypoxia or shock of varying pathogenesis was demonstrated macroenzymatically. It was also demonstrated to be caused by coronary air embolism, and by ectasia of the coronary artery. Myocardial necrosis represents a serious anatomical and functional complication in various clinical disorders and in

surgical cases, which may significantly contribute to the mortality in these conditions.

Normal heart muscle contains substrates, coenzymes and enzymes. It is suggested here that the coenzyme NAD and the enzyme NAD tetrazolium reductase are the first to be depleted from the injured myocardium. This could be of clinical significance in the diagnosis and prognosis of acute myocardial infarction.

The persistence of the tetrazolium dehydrogenase reaction when NAD is added to the incubating medium draws attention to the nature of the enzymatic macroreaction per se. This leads one to consider that the reaction, in fact, depends upon NADH tetrazolium reductase within the tissue. The addition of phenazine methosulphate (PMS) to by-pass the endogenous tetrazolium reductase in the dehydrogenase macroreaction resulted in reduction of nitroblue tetrazolium in the incubating medium and artefactual non-selective staining of the heart. It seems that PMS accelerated the transfer of electrons to the tetrazolium salt in solution rather than in situ.

A non-enzyme histochemical method, phloxine tartrazine (Lendrum, 1947), was used here as an aid in the identification of early human myocardial infarction in microscopic tissue sections. The method revealed areas of recent myocardial damage long before myocardial fibres show any histologic evidence of necrosis. It proved to be more valuable than other conventional histochemical methods at the microscopic level.

The suggestion that waviness of myocardial fibres is an early index of acute myocardial infarction was found here to be unjustified. Such fibres were found in a blind study to be equally distributed between infarcted and control hearts. They were frequently also seen in the autolysed heart.

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NITROBLUE TETRAZOLIUM TEST: EARLY GROSS DETECTION OF HUMAN MYOCARDIAL INFARCTS

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Summary.—The hearts from 81 cases of suspected mycoardial infarction were stained with the nitroblue tetrazolium (NBT) test to show damaged heart muscle in the gross at necropsy. Thirty-seven cases were of stated clinical age less than 12 h and 27 of these were less than 5 h. Seven out of 17 cases under 1 h were negative with NBT, but all other cases showed either focal diminution of staining with the dark blue diformazan or patchy red staining with the monoformazan of NBT. Thus the method may be of diagnostic value at necropsy from 1 h onwards after the time of apparent infarction (stated clinical onset). Satisfactory results were obtained up to 3 days at ambient temperature after death and for longer when the corpse was stored at 4°C.

A MAJOR PROBLEM in pathology is the accurate recognition of early myeoardial infarcts in the gross at necropsy. A number of histochemical and morphological methods have been reported for the microscopic identification of early infarcts, but these are of limited application if the area to be examined cannot be identified and localized with the naked eye. Apart from potassium estimation, the only macroscopic methods at present available depend upon the enzymatic reduction of a suitable redox indicator by normal heart muscle and the focal absence of such staining in infarction (Sandritter and Jestädt, 1957–58; Kolin, Neoral and Kodousek, 1959; Nachlas and Schnitka, 1963). The favoured method depends on loss of dehydrogenase activity detected by nitroblue tetrazolium (NBT). It is not clear in man how soon after infarction enzyme activity is lost from the lesion, but reports on isolated cases have hitherto suggested an interval of 3½–8 h (as above; Knight, 1967; McVie, 1970). In discussion, some observers have suggested an interval of 24 h. This contrasts with intervals of 1½–6 h with induced infarcts in 3 species of experimental animals (Nachlas and Schnitka, 1963; Buss, 1970; Feldman *et al.*, 1976). A further complication has been introduced by the reported loss of activity after 6–36 h of postmortem autolysis at ambient temperature (Nachlas and Schnitka, 1963; Knight, 1967).

The purpose of this communication is to show that the NBT test can become positive as soon as 1 5 h after the onset of infarction and that satisfactory results can be achieved even after 3 days of postmortem storage.

MATERIALS AND METHODS

Hearts were obtained at necropsy from 81 subjects in whom the diagnosis of myocardial infarction had been suspected from the history or established on clinical grounds. A further miscellaneous group of 7 hearts was examined, as well as a group of 10 normal hearts. The clinical history of 37 of these 81 supposed cases of myocardial infarction suggested that occlusion had occurred within 12 h of death. In all but 6 of these recent cases the occlusion was thrombosis of one or more coronary arteries (Table II) The majority of cases were coroner's postmortems; the others were mainly hospital patients who had been admitted for myocardial infarction or investigation of heart disease.

Six groups of histochemical methods were tested for macroscopic staining of the heart:

these included 6 for dehydrogenase; 2 nucleotide (NAD, NADP) tetrazolium reductases, monoamine and cytochrome oxidases, acid phosphohydrolase, aliesterase, leucine aminopeptidase and phosphorylase (Adams, 1967). Of these, the most useful in the gross were the dehydrogenase tests with nitroblue tetrazolium, using either the heart's own (endogenous) substrate or β-hydroxybutyrate. Slices of fresh myocardium were rinsed in cold running water to remove blood, and were then incubated for 20–30 min at 37° in medium containing nitroblue tetrazolium (NBT, Sigma; 50 mg/100 ml) in 0·2M Tris-HCl buffer at ph 7·4, alone or with β-hydroxybutyrate (12·7 g/100 ml) as substrate. Sodium cyanide (500 mg/100 ml) and nicotinamide adenine dinucleotide (NAD, Sigma; 100 mg/100 ml) were added to the incubating medium, the final pH of which was adjusted to 7·1 with 0·2M Tris without HCl. The acetone rinse, used in histochemical practice to remove the red monoformazan, was omitted in this gross test (Adams, 1967). The incubated slice was washed in cold running water and stored in 10% formalin. A block was taken from the mirror-image surface, fixed in 10% formalin and used for routine histology.

RESULTS AND COMMENT

The microscopical criteria for dating infarcts are set out in Table I. Loss of structure and a swollen hyaline appearance are features of infarction from about 5 h after occlusion onwards (Grade B; Mallory, White and Salcedo-Salgar, 1939). Increased eosinophilia of mycoardial fibres also appears at this time (Mallory *et al.*, 1939; Olsen, 1973) and is a particularly valuable and readily detectable sign.

The presence of wavy myocardial fibres (Bouchardy and Majno, 1974) was not in our hands a reliable histological sign of infarction, because normal heart in both man and rat showed such fibres. Likewise, the basic fuchsin method (Lie *et al.*, 1971) gave false positive staining in our hands in some cases and seemed particularly to depend on the extent to which the sections were differentiated.

Normal myocardium stained dark blue with both endogenous and β-hydroxybutyrate NBT methods. Myocardial infarcts of stated clinical age greater than 24 h showed circumscribed areas of near-absent staining, as did areas of laminar subendocardial fibrosis in the inner quarter of the myocardium (Miller, Burchell and Edwards, 1951; Schwartz and Gerrity, 1975; Davies, Woolf and Robertson, 1976), including generalized

TABLE I.—*Criteria for Histological Dating of Myocardial Infarcts*

A.	Presumptive; no eosinophilia; no loss of internal structure	up to 5 h*
B.	Eosinophilia; swollen hyaline appearance to fibres; distortion and some loss of internal structure	5 h onwards*†
C.	Neutrophil polymorph infiltration (more than margination and local perivascular infiltration) plus B	12–24 h onwards*†‡
D.	Clear necrosis and absence of nuclei, plus B and C	1–2 days onwards†‡

* Mallory *et al.* (1939).
† Olsen (1973)
‡ Lodge-Patch (1951).

TABLE II.—*Nitroblue Tetrazolium Staining of Myocardial Infarcts*

Clinical age	NBT staining	Naked eye infarction	Microscopic grade	Thrombosis
Under 1 h	7 transmural	1/7	A, A, A, A, B, B, B*	5/7
	3 zonal	1/3	A, A, B*	3/3
	7 negative	2/7	all A	5/7
1–5 h	6 transmural	1/6	A, B, B, B, B, D*	6/6
	3 zonal	1/3	A, B, B,	3/3
	1 mixed	0/1	B	1/1
5–12 h	8 transmural	5/8	B, B, B, B, B, C, C, D†	7/8
	1 zonal	0/1	C	1/1
	1 mixed	1/1	B	0/1

* With naked eye infarction.
† Necrotic to naked eye.

ischaemia of the papillary muscles. Old scarred infarcts appeared as nearly white fibrous areas. Certain other conditions showed focal loss of myocardial enzyme activity, notably one case each of severe megaloblastic anaemia, congestive cardiomyopathy, rheumatic mitral stenosis and calcific aortic stenosis.

Thirty out of 37 recent cases of stated clinical age less than 12 h showed in the gross either focal diminution in dark blue (diformazan) staining with NBT or patches of pink or red staining by the monoformazan of NBT (Fig. 1 and 2). The presence of the red monoformazan (reduced form of the contaminating monotetrazole of NBT) is usually regarded as evidence of only weak dehydrogenase activity (Adams, 1967). The NBT test was positive in 20/30 cases (Table II) in which infarction could not be seen with the naked eye and where either only pre-inflammatory microscopic signs were present (Figs. 3 and 4; Grade B in Table I)

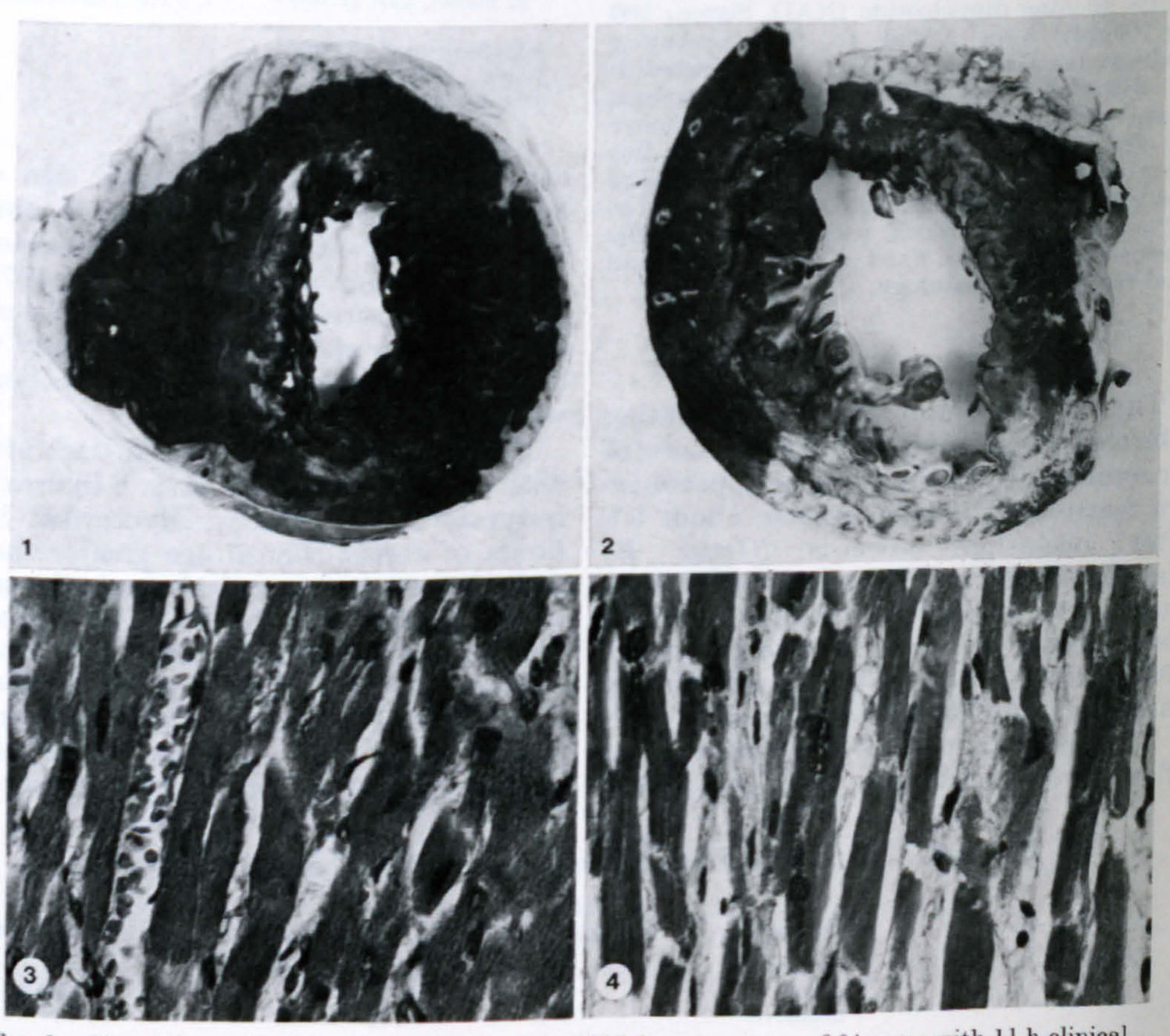

FIG. 1.—Heart slice stained by β-hydroxybutyrate-NBT from a woman of 64 years with 1½ h clinical history. Occlusion of trunk of right coronary artery by recent thrombus; recent mottled postero-septal infarct; also small fibrous scar in anterior septum (top).

FIG. 2.—Heart slice stained by NADPH-tetrazolium (NBT) reductase from a woman of 65 years with clinical history of less than 1 h. Occlusion of right coronary artery by recent thrombus; recent mottled infarct of septum; older fibrous infarct in posterior wall (bottom).

FIG. 3.—Eosinophilic infarcted area in man of 26 years with 4½ h clinical history. Note swelling of some fibres, oedema and some loss of structure. H. & E. ×232.

FIG. 4.—Eosinophilic infarcted area from same case as Fig. 1. Note oedema, fractures and loss of structure in some fibres. H. & E. ×232.

or histological evidence was absent (Grade A in Table I). Seven out of 17 cases of clinical age under 1 h were negative with NBT (Table II).

It is likely that the clinical age was wrong and underestimated infarct age in a proportion of our cases. However, it can be stated that the NBT test was positive in 6/10 cases with a clinical age of under 1 h and before eosinophilia and fibrillar changes could be detected under the microscope (*i.e.* histologically less than 5 h; Grade A in Table I). Even if the clinical age of 1 h was wrong in all 6 of these cases, they would not seem from the histological evidence to be older than 5–12 h. One case deserves particular comment: coronary air embolism had complicated the course of transfusion 12 h before death. The heart in this case was clearly positive with the NBT test and histologically was of Grade B.

Our results suggest that the NBT method is of diagnostic use in the gross from 1–5 h onwards. This conclusion is consistent with observations in the dog (Nachlas and Schnitka, 1963), but is somewhat shorter than the $3\frac{1}{2}$–8 h interval previously reported in isolated cases of human myocardial infarction (refs. in Introduction). The superior results we have obtained may be related to our use of cyanide to divert electron transfer away from the cytochrome oxidase system towards the tetrazolium salt. Moreover, the addition of coenzyme (NAD) to the "endogenous" medium is a modification not used in previous studies on gross-staining of infarcts and would seem to have contributed towards development of colour.

Three normal human hearts were kept at ambient temperature between 18 and 25° for periods of 24–72 h. They were sampled at intervals: NBT staining of slices with or without added substrate was not significantly diminished over the 72-hour period. Seven hearts were kept at 4° for a week and these, likewise, showed no loss of enzyme activity. These results contrast with those of Nachlas and Schnitka (1963), who found that post-mortem autolysis prevented an adequate reaction with endogenous substrate by about 6 h.

An important advantage of the NBT gross test is the information gained about the location and extent of the infarct. Without some gross definition of the lesion, one or two random blocks for histology might well fail to detect a medium-sized infarct.

The conclusions from this study are:

(1) the NBT test for gross detection of myocardial infarction shows focally diminished or altered staining as early as 1–5 h after the stated clinical onset in man;
(2) human material can be tested for at least 3 days after death and for longer if stored at 4°;
(3) results with endogenous (no added) substrate are, in practice, as satisfactory as with added substrate.

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The non-specific nature of the myocardial wavy fibre

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The non-specific nature of the myocardial wavy fibre

Wavy myocardial fibres were found in about half each of a series of 28 normal and 31 infarcted human hearts, as well as in the normal heart of an infant. Such fibres were also seen in rather more than half of a series of normal rat hearts. Thus, the wavy fibre is not a specific feature of acute ischaemic heart disease. Some experimental evidence was obtained that patchy loss or preservation of ATP promotes the formation of wavy fibres.

Keywords: myocardium, wavy fibre, infarction, enzymes

Introduction

It has previously been reported that the presence of wavy myocardial fibres is a characteristic and diagnostic feature of myocardial infarction (Bouchardy & Majno 1974). Infarcted myocardial muscle uses up its energy-rich substrates so that it would be expected to take up a relaxed extended state. This would lead to an excess 'slack' or length in some of the muscle fibres surrounding the infarcted zone, and such fibres would perforce become wavy.

However, in three recent studies on myocardial infarction, considerable suspicion has arisen about the specificity and reliability of the wavy fibre as a histopathological index of myocardial infarction (Rose, Opie & Bricknell 1976, Sakurai 1977, Derias & Adams 1978). For this reason we decided to examine the muscle fibres in normal human and rat heart to see how often wavy fibres are present in the normal intact organ.

Materials and methods

Twenty-eight hearts were obtained from subjects with no clinical history of angina,

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infarction or hypertension. The subjects were aged 18–77 years. None of them showed evidence at autopsy of myocardial infarction or of coronary occlusion by thrombosis or stenosing atheroma; in none of them was there a severe grade of coronary atherosclerosis. The heart was also obtained from an infant aged 6 weeks, who had died with no disease of the cardiovascular system. Thirty-one hearts were obtained from subjects aged 25–84 years, in whom post-mortem and microscopical evidence of myocardial infarction had been obtained (Derias & Adams 1978).

Heart muscle was also obtained from 16 normal albino rats.

Blocks of human heart muscle were taken into 10% formol-saline, embedded in paraffin and stained by haematoxylin and eosin. The mirror-image slice was stained in the gross with nitroblue tetrazolium with added coenzyme I (NAD; nicotinamide adenine dinucleotide) but no added specific substrate. This particular coenzyme-enriched non-specific dehydrogenase incubating medium has been found to give optional results for the gross detection of myocardial infarction (Derias & Adams 1978).

The stained sections from the series of 31 infarcted hearts were examined by the first author and areas of infarction, which were recognizable under the microscope, were masked by applying black ink to the coverslip. The sections from the 28 normal hearts were treated in a similar way to mimic the infarction series. Both series were randomized by the first author and were then examined for wavy fibres by the second author.

Twelve normal rat hearts were used to study the effects of adenosine-5-'triphosphate (ATP) and 2,4-dinitrophenol (DNP) on the formation of wavy fibres. It was thought that patchy absorption of ATP might promote patchy muscle contraction and intervening areas of wavy fibres. By contrast, uncoupling of oxidative phosphorylation by DNP might be expected to cause uniform relaxation and an absence wavy fibres.

Adjacent cross-sectional slices from normal rat hearts were incubated for 24–48 hours either in 0.1M ATP (Sigma) in 0.15M saline buffered to pH 7.4 with 20 mM tris or in 0.1M DNP (Sigma) in saline buffered as above. A further series of slices from eight rats hearts were incubated for 2 hours in 0.01M solutions of ATP or DNP in buffered saline. After incubation all slices were fixed in 10% formol-saline and then processed into paraffin.

Results and comment

Wavy fibres were seen in about half the normal and half of the infarcted human hearts (Table 1); they were even present in the heart of the 6-week-old infant (Figure 1) and were prominent in older subjects (Figure 2). Such fibres were indistinguishable from those around areas of myocardial infarction (Figure 3): primary, secondary and tertiary-order wavy fibres were seen, as described by Bouchardy & Majno (1974). Wavy fibres were also seen in the myocardium in eleven out of sixteen normal rats examined (Figure 4).

The 'blind' examination of the partly masked sections from the series of 28

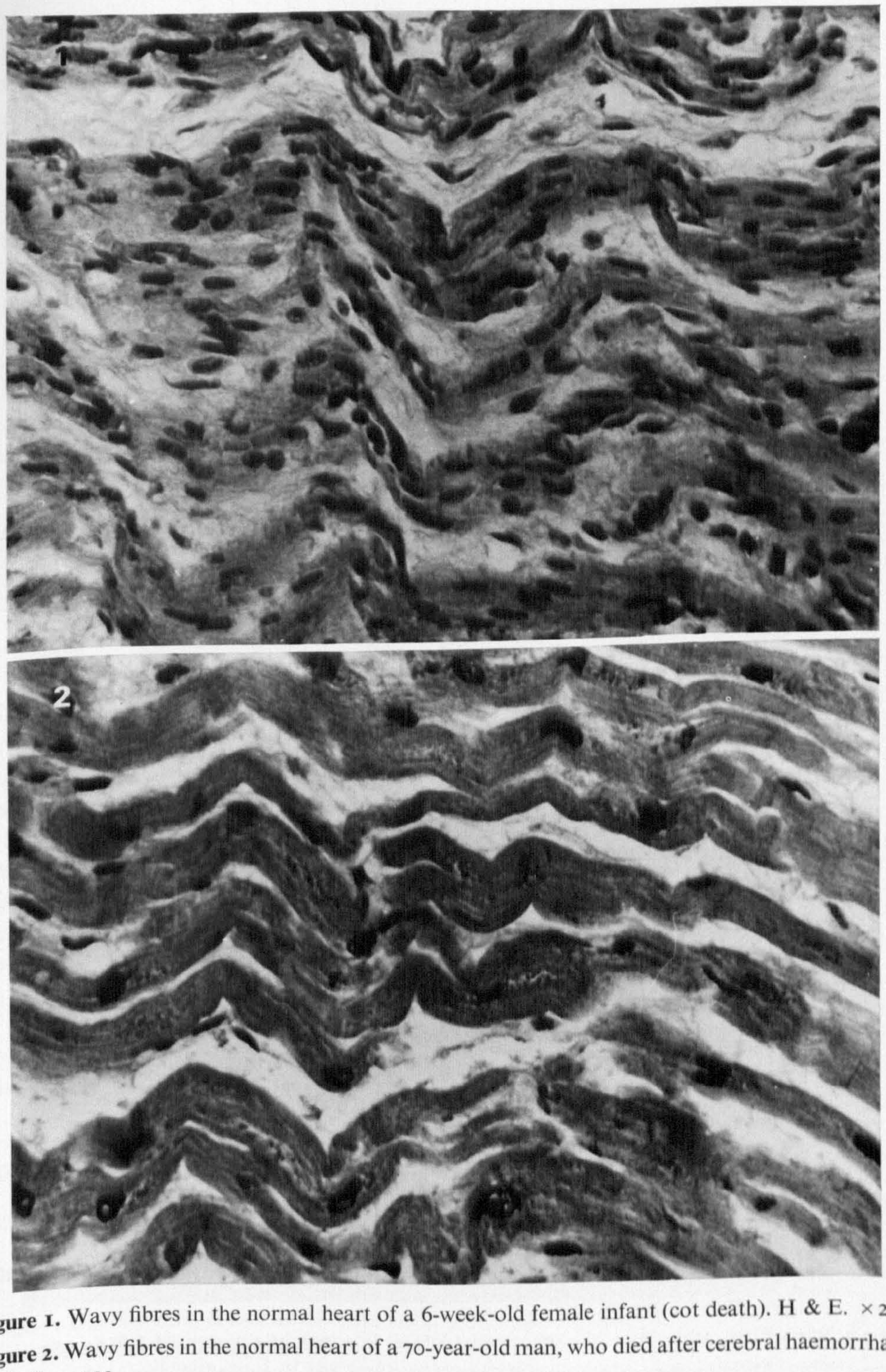

Figure 1. Wavy fibres in the normal heart of a 6-week-old female infant (cot death). H & E. ×240.

Figure 2. Wavy fibres in the normal heart of a 70-year-old man, who died after cerebral haemorrhage. H & E. ×320.

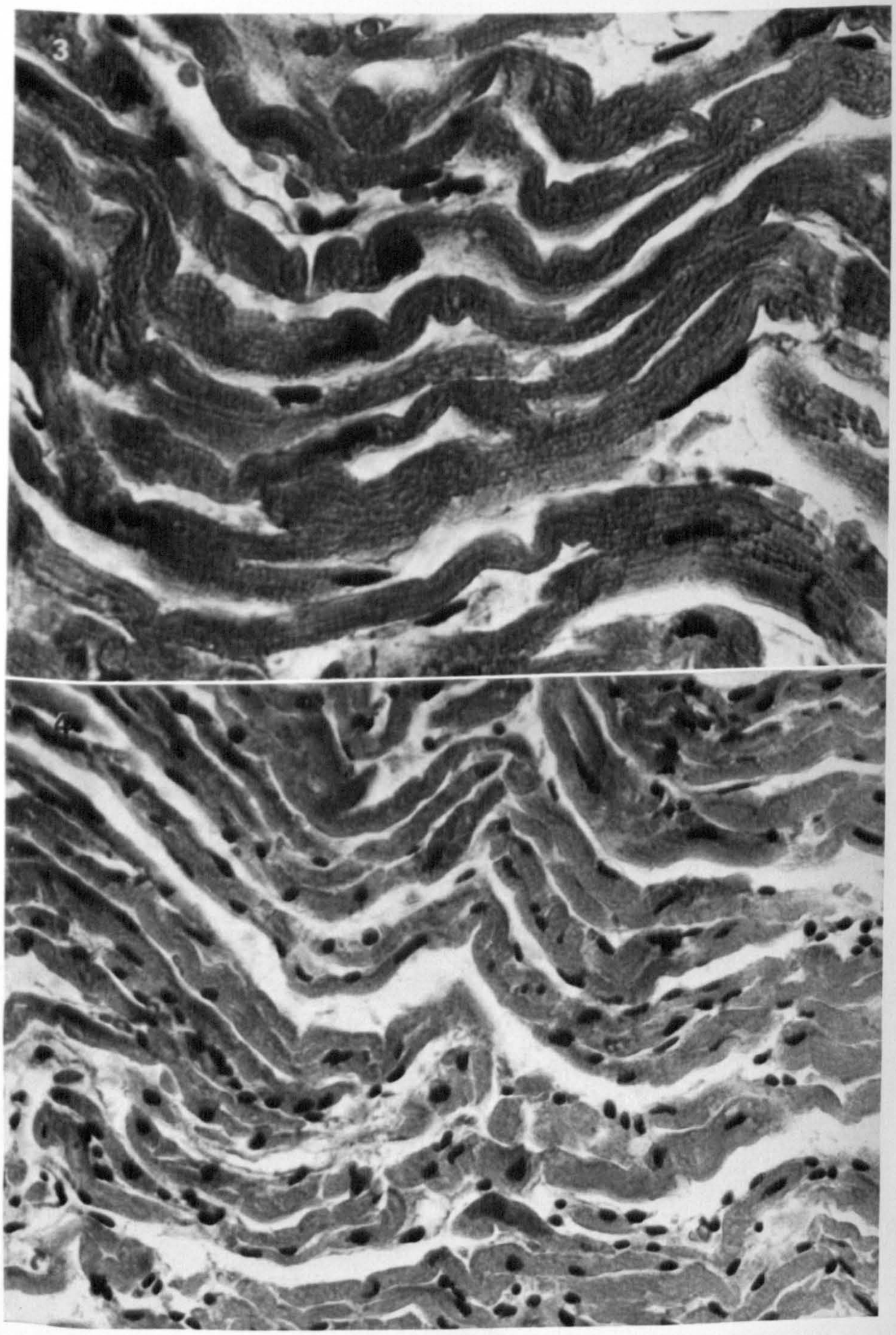

Figure 3. Wavy fibres at the edge of a myocardial infarct in a man of 52 years. H & E. ×320.
Figure 4. Wavy fibres in normal rat heart. H & E. ×240.

normal and 31 infarcted human hearts appeared to be truly blind, because the observer's guess as to which was which seemed to be quite random. Even 'fragmentation' of muscle fibres in an infarcted heart was matched by a similar finding in a normal heart. Likewise, the two cases with the most prominent wavy fibres were, respectively, from a normal and an infarcted heart.

The nitroblue tetrazolium reaction was positive in the mirror-image slice next to the samples of normal human heart taken for histology. This indicated an absence of established ischaemic damage in these normal samples. It is apparent that the wavy fibre is a non-specific feature and, although found at the edge of infarcted areas, is also seen in the normal myocardium.

Table 1. Incidence of wavy fibres in normal and infarcted human myocardium

Number of wavy fibres	Normal hearts	Infarcted hearts
None or equivocal	13	16
Moderate	9	7
Many	6	8
Totals	28	31

As mentioned above, the presence of wavy fibres may reflect the tonicity of myocardial fibres at the time of fixation. If myofibrillar ATP is either fully preserved or fully exhausted, the fibres would, respectively, all be contracted or all relaxed. Hence, wavy fibres would be absent. However, if ATP is patchily preserved or lost, then conditions would be appropriate for forming wavy fibres. The incubation studies with ATP and DNP support this explanation: four out of four rat hearts showed wavy fibres with ATP at the 0.1M concentration, but none of four with 0.1M DNP; six of eight rat hearts showed wavy fibres with ATP at the 0.01M concentration, but only three out of eight with 0.01M DNP. It would seem that patchy uptake of ATP promotes wavy fibres, whereas DNP—particularly at the stronger concentration—inhibits the process.

Our tentative conclusion is that wavy fibres are caused by uneven contraction of myocardial fibres brought about by patchy preservation or loss of ATP. Such changes may result from infarction or from post-mortem autolytic changes.

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